

Original Research Article

MicroRNA-421 protects against chronic intermittent hypoxia-induced vascular endothelial cell injury by targeting TLR4

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Abstract

Purpose: To investigate the role of miR-421 in endothelial cell injury caused by chronic intermittent hypoxia (CIH), and to unravel the mechanism of action.

Methods: A rat aortic endothelial cell model of CIH was established by 18-h exposure to hypoxic treatment. Cell viability was evaluated by MTT while cell apoptosis was determined by flow cytometry. Cellular reactive oxygen species (ROS) levels were assessed by cellular reactive oxygen species (ROS) assay kit. The mRNA and protein levels were also determined. Sprague Dawley rats were used to establish a rat CIH model over a 6-week hypoxic exposure. The degree of lung and renal injuries in the rats were observed by HE staining.

Results: MiR-421 was downregulated and toll-like receptor 4 (TLR4) upregulated in CIH cells compared to control cells. Treatment of CIH cells decreased their viability, increased cellular ROS levels, promoted cell apoptosis, and caused changes in protein levels of apoptosis- and inflammation-related genes. However, miR-421 mimics reversed these results caused by CIH treatment. Dual luciferase reporter assay verified that TLR4 was targeted by miR-421. Moreover, TLR4 overexpression suppressed the protective effect of miR-421 on CIH cells. Finally, miR-421 agomiR inhibited CIH-induced TLR4 upregulation in rats. Histopathological examinations confirmed that miR-421 agomiR inhibited CIH-induced injury and collagen deposition in rat lungs and kidneys.

Conclusion: MiR-421 protects vascular endothelial cells against CIH-induced injury in rats by targeting TLR4, which may provide a biomarker for the diagnosis and treatment of CIH-induced injury.

Keywords: Obstructive sleep apnea syndrome, MiR-421, TLR4, Chronic intermittent hypoxia, Vascular endothelial cells

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INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is a common condition resulted from recurrent episodes of complete or partial obstruction in upper airway [1]. It may be caused by obesity,

advanced age, male gender, alcohol abuse, and nasal obstruction [2]. It is well-known that OSAS can cause damage to multiple organs and systems, including the cardiovascular system, nervous system, lung, and kidney, [3-5]. Importantly, chronic intermittent hypoxia (CIH) is

a characteristic of OSAS [4] and a key factor of oxidative stress that induce the release of reactive oxygen species (ROS), which subsequently results in systemic inflammation [1]. Previous studies confirmed that CIH is responsible for the dysfunction and injury of endothelial cell [6,7].

MicroRNAs (miRNAs) consist of about 22 nucleotides that can modulate the expression of target genes [7-9]. They play key roles in various diseases, such as cancers, diabetes, and cardiovascular diseases [8,10,11]. Many miRNAs are involved in the development of OSAS [5,12]. For example, miR-664a-3p expression is downregulated in OSAS patients [13]. It has also been reported that miR-630 may play important roles in OSAS-induced endothelial dysfunction [14]. The suppression of miR-30a inhibits CIH-induced endothelial cell damage [6]. In particular, miR-421 plays key roles in various diseases such as cancer, epilepsy, and nonalcoholic fatty liver disease (NAFLD) [15-17]. Moreover, in human umbilical vein endothelial cells, it has been reported that miR-421 interacts with plasminogen activator inhibitor [18].

However, the relationship between miR-421 and CIH-induced endothelial cell damage is unclear. Besides, previous reports demonstrated that TLR4 had important roles in the pathogenesis of CIH-induced injury [19,20]. Thus, to determine whether miR-421 protects endothelial cells against CIH-induced damage via targeting TLR4, a series of *in vivo* and *in vitro* experiments were performed.

EXPERIMENTAL

Plasmid construction, miRNAs, and transfection

The miR-421 mimics (miR-421), miR-NC, miR-421-inhibitor, NC-inhibitor, miR-421 agomiR, and NC agomiR were from RiboBio (Guangzhou, China).

TLR4 cDNA was amplified (forward: 5'-AGAAGAGCTGCAGCACCTGGATTT-3'; reverse: 5'-GATGAAATTGGAATGAAGACCTCTCA-3') and cloned into the pcDNA3.1vector (Promega, Madison, WI, USA) to construct the TLR4 overexpression plasmid (pcDNA3.1-TLR4).

Rat aortic endothelial cell model

After synchronization, rat aortic endothelial cells were grouped as follows: control, CIH, CIH+miR-NC, CIH+miR-421 and CIH+miR-

421+pcDNA3.1-TLR4 group. Cells in control group were kept in serum-free culture under normoxic condition. In the CIH group, cells were treated with 21 to 1 % of O₂ every 15 min in a chamber (Proox C21, Biospherix, Parish, NY, USA) for 18 h. In the CIH+miR-NC, CIH+miR-421 group, and CIH+miR-421+pcDNA3.1-TLR4 groups, cells were co-transfected with miR-NC, miR-421 mimics, miR-421 mimics and plasmid pcDNA3.1-TLR4 by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 48 h, cells were harvested and exposed to 18-h CIH as described above.

Animal model

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committees (IACUCs) of the United States [21] and approved by the institutional Animal Ethical and Welfare Committee (approval no. 2019-802).

Forty-five Sprague Dawley rats (male, 200±20 g; 6-week-old; from the Laboratory Animal Research Center of Fourth Military Medical University) were grouped as follows: sham, CIH+NC agomiR, and CIH+miR-421 agomiR (n=15 in each group). Rats in the CIH+NC agomiR and CIH+miR-421 agomiR groups were kept in the chamber (Oxycycler model A84; Biospherix) for 30 cycles/h for 8 h daily (from 9 am to 5 pm) for 6 weeks. In each 2-min cycle, oxygen concentration was dropped to 8% for 30sec, maintained for 50sec, and then increased to 20% for 40sec. Sham rats were placed in the chamber with 20% oxygen throughout the experiment. Besides, rats in the CIH+NC agomiR and CIH+miR-421 agomiR groups were injected through the tail vein with NC agomiR or miR-421 agomiR at a concentration of 15 mg/kg/day twice a week. At week 6, blood was collected through cardiac puncture before euthanization. Lungs and kidneys were collected for further analysis.

Cellular ROS assay

Cellular ROS levels were assessed with a 2', 7'-dichlorofluorescein diacetate cellular ROS detection assay kit (cat.no: ab113851, Abcam, Cambridge, UK).

MTT assay

Cells (5 × 10³) were seeded into plates (12-well) and incubated (2 days, 37°C). Then, MTT (Sigma, St. Louis, MO, USA) was added and incubated (4 h). Next, the supernatant was replaced with dimethyl sulfoxide, and absorbance measured spectrophotometrically at 490 nm.

Flow cytometry analysis

Cells from different groups were washed with phosphate-buffered saline and then stained (Annexin V-fluorescein isothiocyanate and propidium iodide) for 10 min. Apoptotic cells were examined using a flow cytometer (Beckman Coulter, Brea, CA, USA).

Quantitative polymerase chain reaction (qPCR)

The RNA from cells was extracted and cDNA was synthesized. qPCR was conducted with SYBR Green quantitative RT-PCR kits (Sigma). Relative expression levels were calculated as ratios normalized against those of GAPDH, using the following primer sequences:

TLR4	forward:	5'-
		CCTTCGAGAAGCTCTCACGAC-3';
TLR4	reverse:	5'-CCGTTGGCGTAAAGGATGC-
		3';
miR-421	forward:	5'-
		TGGAAACTTCCCGAAGAAC-3';
miR-421	reverse:	5'-
		GGGATCAACAGACATTAATT-3';
GAPDH	forward:	5'-
		TCATTGACCTCAACTACATGGTTT-3';
GAPDH	reverse:	5'-
		GAAGATGGTGATGGGATTTTC-3'.

Dual luciferase reporter assay

The 3'-UTR of TLR4 harboring wide-type or mutant putative miR-421 binding sites were amplified to construct TLR4 3'-UTR (WT) and TLR4 3'-UTR (MUT) plasmids. Cells were transfected with miR-421 mimics or miR-NC, and TLR4 3'-UTR (WT) or TLR4 3'-UTR (MUT) plasmid. Luciferase activities were measured at 48 h.

Histopathological examination

Lungs and kidney tissue were fixed (10% formalin) and embedded (paraffin). Hematoxylin-eosin (HE) or Masson staining was performed on 3- μ m serial sections. All sections were observed under an optical microscope.

Western blot

Proteins were extracted from cells and equal amount of protein were separated and transferred to nitrocellulose membranes. Then, membranes were incubated with primary antibodies (pro-caspase 3, 1:500; pro-caspase 9, 1:500; cleaved-caspase 3, 1:400; cleaved-

caspase 9, 1:400; Bax, 1:200; Bcl-2, 1:400; interleukin (IL)-1 β , 1:500; IL-6, 1:200; tumor necrosis factor (TNF)- α , 1:200; TLR4, 1:300; β -actin, 1:200) for 16 h at 4°C, including. After washing with TBST, blots were incubated with secondary antibody at 1:400 for 1 h. The antibodies were purchased from Abcam. Bands were visualized and analyzed.

Enzyme-linked immunosorbent assay (ELISA)

Cellular IL-1 β , IL-6, and TNF- α levels were detected using Rat ELISA Kits (ab100704, ab100713 and ab108910, respectively; Abcam).

Statistical analysis

Data are presented as mean \pm SD, and each experiment consisted of at least three replicates per test. Differences between groups were assessed using one-way analysis of variance. The statistical significance threshold for all data was $p < 0.05$.

RESULTS

CIH treatment downregulated miR-421 and upregulated TLR4 in rat aortic endothelial cells

The viability of rat aortic endothelial cells was decreased in CIH group (Figure 1A, $p < 0.05$). Figure 1B showed that CIH group had lower miR-421 levels than control group ($p < 0.001$). However, the mRNA and protein expression of TLR4 were upregulated in CIH group (Figure 1C and D, $p < 0.01$).

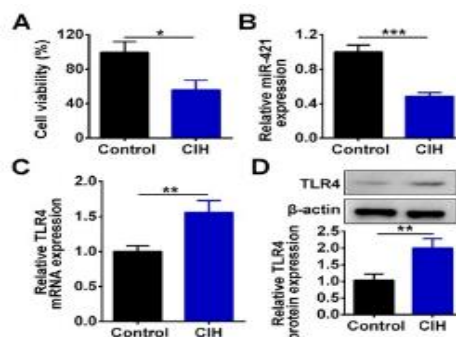


Figure 1: Effect of CIH treatment on the expressions of MiR-421 and TLR4 in rat aortic endothelial cells. (A) Cell viability was determined by MTT assay. The mRNA levels of miR-421 (B) and TLR4 (C) were evaluated by qPCR. D. TLR4 protein expression level was analyzed by western blot; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

MiR-421 overexpression inhibited CIH-induced apoptosis and inflammation in rat aortic endothelial cells

Figure 2 A showed that miR-421 levels were decreased in CIH group ($p < 0.01$). However, miR-421 mimics reversed CIH-induced downregulation of miR-421 (Figure 2 A, $p < 0.001$). The viability of rat aortic endothelial cells was decreased in the CIH group (Figure 2 B, $p < 0.01$). However, miR-421 mimics increased the reduction in cell viability caused by CIH-treatment (Figure 2 B, $p < 0.05$). Moreover, CIH treatment significantly increased cellular ROS levels (Figure 2 C, $p < 0.001$), whereas miR-421 mimics abolished this phenomena (Figure 2 C, $p < 0.01$). Treatment of CIH also promoted cell apoptosis (Figure 2 D, $p < 0.001$), and was suppressed by miR-421 mimics (Figure 2 D, $p < 0.05$). In addition, the results showed that the protein levels of pro-caspase3, pro-caspase9, and Bcl-2 were significantly decreased, whereas cleaved caspase 3, cleaved caspase 9, Bax, IL-1 β , IL-6, and TNF- α were significantly upregulated in CIH group (Figure 2 E, $p < 0.001$ for cleaved caspase 9, Bax, IL-1 β , and Bcl-2; $p < 0.01$ for other genes). In contrast, miR-421 mimics reversed CIH-induced changes in protein levels of apoptosis- and inflammation-related factors (Figure 2 E, $p < 0.01$). Besides, ELISA assay confirmed that miR-421 mimics significantly inhibited the CIH-induced enhancement of inflammation factors (Figure 2 F, $p < 0.05$ for IL-6, and $p < 0.01$ for IL-1 β and TNF- α).

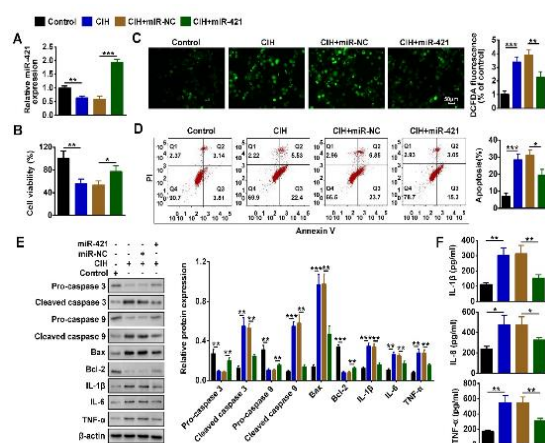


Figure 2: MiR-421 overexpression inhibited CIH-induced apoptosis and inflammation in rat aortic endothelial cells A. qPCR assay of miR-421 level. B. MTT assay of cell viability. C. The measurement of cellular ROS levels. D. Flow cytometry assay of cell apoptosis. E. Western blot assay of protein levels. F. ELISA analysis of IL-1 β , IL-6, and TNF- α ; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Identification of TLR4 as a miR-421 target gene

TargetScan assay predicted that there were putative target sites of miR-421 in the TLR4 3'-UTR (Figure 3 A). Dual luciferase reporter assays showed that miR-421 mimics inhibited luciferase activity in cells harboring WT TLR4 3'-UTR ($p < 0.01$), whereas had little effect on the luciferase activity of cells bearing MUT TLR4 3'-UTR (Figure 3 A). In rat aortic endothelial cells, miR-421 level was increased by transfection of miR-421 mimics group and was decreased by transfection of miR-421-inhibitor (Figure 3B, $p < 0.001$). Besides, miR-421 mimics inhibited TLR4 mRNA (Fig 3C) and protein expression (Figure 3 D) in endothelial cells ($p < 0.01$). However, miR-421 inhibitor treatment promoted TLR4 mRNA (Figure 3 C, $p < 0.05$) and protein (Figure 3 D, $p < 0.01$) expression.

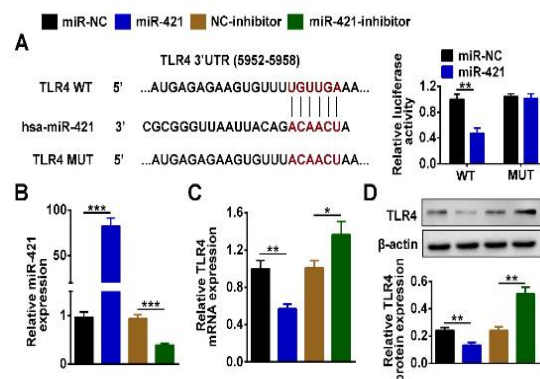


Figure 3: TLR4 was a target gene of miR-421 in rat aortic endothelial cells A. Schematic diagram of miR-421 binding sites in the TLR4 3'-UTR. Dual luciferase reporter assay was conducted 48 h after transfection. The relative expression levels of miR-421 (B) and TLR4 (C) were measured using qPCR. D. The relative TLR4 protein level was measured using western blot; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

TLR4 overexpression reversed the protective effects of miR-421 on rat aortic endothelial cells

Figure 4 A shows that miR-421 mimics increased the viability of rat aortic endothelial cells under CIH stress compared to miR-NC ($p < 0.05$), whereas co-transfection of pcDNA3.1-TLR4+miR-421 mimics reversed this result ($p < 0.01$). Fig 4B demonstrated that miR-421 mimics inhibited CIH-induced increase in cellular ROS ($p < 0.05$), whereas co-transfection of pcDNA3.1-TLR4+miR-421 mimics reversed this result ($p < 0.01$). Compared to miR-NC, miR-421 mimics inhibited cell apoptosis caused by CIH stress, whereas co-transfection of pcDNA3.1-TLR4+miR-421 mimics suppressed the inhibitory

effect of miR-421 mimics on cell apoptosis (Figure 4 C, $p < 0.01$).

Western blotting revealed that miR-421 mimics downregulated TLR4 protein expression in CIH-treated endothelial cells, whereas co-transfection of pcDNA3.1-TLR4 plasmid increased miR-421 mimics-induced downregulation of TLR4 (Figure 4 D, $p < 0.001$). Moreover, co-transfection of pcDNA3.1-TLR4 plasmid and miR-421 in CIH group reversed miR-421 mimics-mediated increases in protein levels of pro-caspase 3 ($p < 0.001$), pro-caspase 9 ($p < 0.001$), and Bcl-2 ($p < 0.01$) and the decreases in protein levels of cleaved caspase 3 ($p < 0.01$), cleaved caspase 9 ($p < 0.01$), Bax ($p < 0.01$), IL-6 ($p < 0.05$), TNF- α ($p < 0.05$), and IL-1 β ($p < 0.001$, Figure 4 D). ELISA assay confirmed that TLR4 overexpression inhibited the miR-421 mimics-mediated decrease in inflammatory factor levels (Figure 4 E, $p < 0.01$).

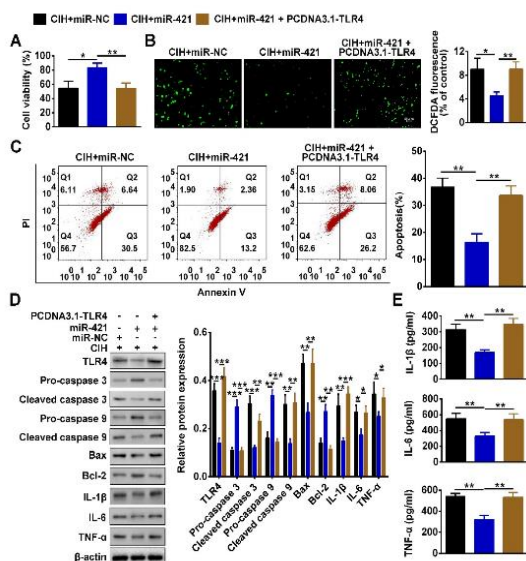


Figure 4: TLR4 overexpression reversed the protective effects of miR-421 in rat aortic endothelial cells. A. Cell viability was detected by MTT assay. B. Cellular ROS levels were assessed by cellular ROS detection assay. C. Cell apoptosis was analyzed using flow cytometry. D. Protein expression levels were assessed by western blot. E. The levels of IL-1 β , IL-6, and TNF- α were measured by ELISA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

MiR-421 inhibited CIH-induced injury in rat lungs and kidneys

As compared to the sham group, the miR-421 level in the CIH+NC agomiR group was decreased to 51 % (Figure 5 A, $p < 0.01$), whereas miR-421 level in CIH+miR-421 agomiR group was increased as compared to CIH+NC

agomiR group (Figure 5 A, $p < 0.001$). Rats in the CIH+NC agomiR group had higher mRNA and protein levels of TLR4 than sham group (Figure 5B and C, $p < 0.001$ and $p < 0.01$). However, rats in the CIH+miR-421 agomiR group had lower levels of TLR4 mRNA and protein than CIH+ NC agomiR group (Figures 5 B and C, $p < 0.001$ and $p < 0.05$, respectively). HE staining showed that the degree of lung injury and renal injury in the CIH+NC agomiR group was higher than sham group, whereas miR-421 agomiR injection ameliorated CIH-induced lung and renal injury (Figure 5 D). Importantly, miR-421 agomiR attenuated CIH-induced collagen deposition in lung and renal tissues (Figures 5 D and E).

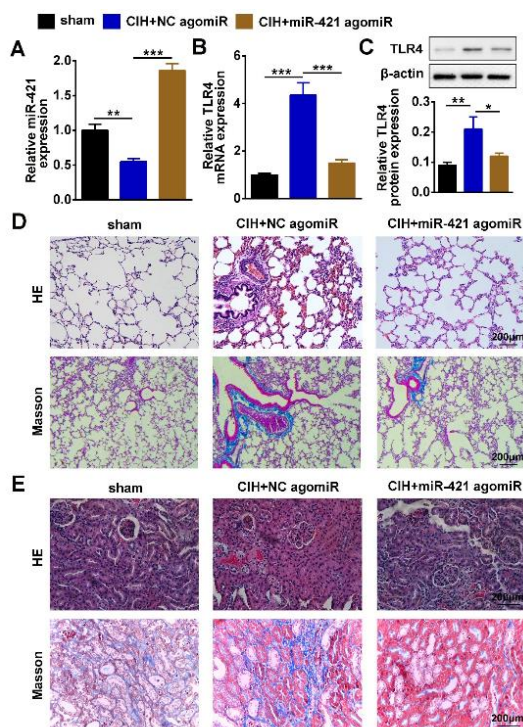


Figure 5: MiR-421 inhibited CIH-induced injury in rat lungs and kidneys. A. Relative miR-421 levels in serum as analyzed using PCR. B-C. Relative TLR4 mRNA (B) and protein (C) levels in serum were detected by qPCR and western blot. The assessments of lung (D) and renal (E) injury were conducted in H&E- and Masson-stained sections; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

DISCUSSION

It is well accepted that OSAS is chronic respiratory disease that can induce CIH, which may result in endothelial dysfunction and multiple organ damage [3,4,6]. Many miRNAs, such as miR-664a-3p, miR-630, and miR-30a, play important roles during the development of OSAS[6,13,14]. In this study, CIH-treated

endothelial cells had lower viability and decreased miR-421 levels compared to control cells, suggesting that miR-421 may exert protective effects on CIH-treated endothelial cells. To verify it, endothelial cells were transfected with miR-421 mimics and then subjected to CIH. Oxidative stress can be stimulated by CIH to trigger systemic inflammation and endothelial dysfunction [1]. Therefore, cell viability, apoptosis ratio, ROS levels, protein expression levels of apoptosis- and inflammation-related genes were assessed. Compared to control group, CIH treatment decreased cell viability, increased cellular ROS levels, promoted apoptosis, caused changes in the protein levels of apoptosis-related genes, and increased inflammatory factor levels. However, miR-421 mimics inhibited these CIH-induced phenomena.

MiRNAs can inhibit transcript expression via complementary base pairing [7]. Previous studies have characterized several direct targets of miR-421, including PDCD4 and caspase-10 in breast cancer [17,22], claudin-11 in gastric cancer [23], and sirtuin 3 in NAFLD [16]. In this study, TargetScan analysis and luciferase reporter analysis identified that TLR4 as a novel miR-421 target. MiR-421 mimics suppressed TLR4 mRNA and protein expression in endothelial cells, whereas miR-421 inhibitor promoted TLR4 mRNA and protein expression. This is the first evidence showing that miR-421 targets the TLR4 3'-UTR and regulates the TLR4 expression in endothelial cells.

Toll-like receptor 4 (TLR4) is a type I transmembrane protein [24], and it has important roles in the development of CIH-induced injury of various organs and systems [19,20,25-27]. For example, Yang *et al* found that TLR4 signaling contributed to CIH-induced pulmonary inflammation [25]. Zeng and colleagues showed that TLR4 signaling promoted CIH-induced atherosclerosis [26]. Zhang *et al* demonstrated that TLR4 deficiency attenuated CIH-mediated renal dysfunction in mice [27].

Consistent with previous reports, TLR4 was upregulated in CIH cells compared to control cells in this study, which suggested that the protective effects of miR-421 on CIH cells may be associated with TLR4 upregulation. To test it, endothelial cells were transfected with miR-421 mimics and TLR4 overexpression plasmid. *In vitro* experiments showed that co-transfection of TLR4 overexpression plasmid suppressed the protective effects of miR-421 on CIH cells. These results suggest that miR-421 protected endothelial cells against CIH-induced injury by

targeting TLR4. Conversely, it is well-known that TLR4 may initiate inflammatory responses via at least two major signaling pathways, including myeloid differentiation factor 88 (MyD88)-independent and -dependent pathways [24,25]. Further experiments are needed to further determine whether these two TLR4 signaling pathways is involved in the role of miR-421 in CIH.

The rat CIH model was established by imitating the state of OSAS. Consistent with *in vitro* experiments, miR-421 overexpression inhibited TLR4 mRNA and protein expression in CIH mice compared to control. Moreover, histopathological analysis demonstrated that miR-421 overexpression attenuated CIH-induced damage to the rat lung and kidney.

Thus, miR-421 downregulation and TLR4 upregulation were closely associated with CIH-induced vascular endothelial cells injury. Moreover, miR-421 had protective effects against CIH-induced damage via targeting TLR4. Restoring the function of miR-421 may be a potential strategy for suppressing CIH-induced damage. In addition, these results suggest that TLR4 may be a novel therapeutic target for OSAS.

CONCLUSION

The findings of this study prove that miR-421 downregulation and TLR4 upregulation are closely linked with CIH-induced vascular endothelial cells injury. Moreover, miR-421 protects against CIH-induced vascular endothelial cell injury by targeting TLR4.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. JY and HZ conceived and designed the experiments, JKS

analyzed and interpreted the results of the experiments, SQY performed the experiments.

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