ACTA ABP BIOCHIMICA POLONICA

Vol. 68, No 2/2021
151–158
https://doi.org/10.18388/abp.2020_5397

Regular paper

MiR-490 alleviates sepsis-induced acute lung injury by targeting MRP4 in new-born mice

Jie Lin, Zongze Lin✉ and Liqun Lin

Department of Paediatrics, Ruian People's Hospital, Ruian Ciy, Wenzhou City, Zhejiang Province, 325200, China

The aim of this study was to investigate whether the effects of miR-490 on acute lung injury (ALI) induced by sepsis *in vitro* and *in vivo* were through targeting multi-drug resistance-associated protein 4 (MRP4). MiR-490 agomir/NC agomir was injected into mice before cecal ligation and puncture (CLP). Pulmonary microvascular endothelial cells (PMVECs) were transfected with or without miR-490 agomir/NC agomir/MRP4/empty vector before lipopolysaccharide (LPS) stimulation. Histopathology, injure score, and Wet/Dry (W/D) of lung tissues were assessed. The number of neutrophils, macrophages and total cells, total protein concentration, TNF-α and IL-1β level in bronchoalveolar lavage fluid (BALF) were measured. The levels of caspase-3, Bcl-2, TNF-α, and IL-1β were measured in MPVECs. Dual-luciferase reporter assay was used to analyze the relationship between MRP4 and miR-490. When compared to the sham group, in CLP mice, the alveolar lung tissue showed significantly hyperemic, alveolar collapse, the W/D ratio was increased, and the injury index was increased. The number of neutrophils, macrophages and total cells, total protein concentration, TNF-α and IL-1β levels were significantly increased in BALF from CLP mice. The levels of TNF-α and IL-1β were significantly increased in lung tissue from CLP mice. Overexpression of miR-490 alleviated lung injury caused by CLP and inhibited inflammation in mice. The levels of TNF-α, IL-1β and caspase-3 were significantly increased, but the level of Bcl-2 was significantly decreased in MPVECs treated with LPS compared to the control group. Overexpression of miR-490 also reversed the increase of TNF-α, IL-1β, cleaved caspase-3 and Bcl-2 caused by LPS in MPVECs. Dual-luciferase reporter assay confirmed that the target gene of miR-490 was MRP4. Besides, overexpression of MRP4 upregulated TNF-α, IL-1β, and cleaved caspase-3, but downregulated the increase of Bcl-2 induced by miR-490 agomir transfection. These data suggested that miR-490 could relieve sepsis-induced acute lung injury in neonatal mice *via* targeting MRP4.

Keywords: miR-490, acute lung injury, sepsis, MRP4

Received: 19 June, 2020; revised: 07 September, 2020; accepted: 24 November, 2020; available on-line: 22 March, 2021

✉e-mail: zongze_lin@163.com

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; CLP, cecal ligation and puncture; MRP4, multi-drug resistance-associated protein 4; LPS, lipopolysaccharide; PMVECs, pulmonary microvascular endothelial cells; W/D, Wet/Dry

INTRODUCTION

Sepsis, a life-threatening disease, is characterized by multiple organ failure and is a common condition in the intensive care units (ICU), especially in pediatric intensive care units (PICU) (Zhang *et al.,* 2012; Sagy *et al.,* 2013; Shin *et al.,* 2018). There are more than 18 million cases of severe sepsis worldwide annually and the mortality rate is still as high as 30–70% (Aziz *et al.,* 2018). Therefore, it is essential to better understand the pathogenesis and develop new treatments for sepsis.

The lung is the most vulnerable target organ in patients with sepsis, and acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) can occur early in patients with sepsis (Ning *et al.,* 2017). ALI is characterized by an over-activated inflammatory reaction in the lungs that leads to severe damage to the body's gas exchange function, damaged alveolar-capillary vascular barriers, and pulmonary edema (Amin & Rahmawati 2017). Pulmonary microvascular endothelial cells (PMVECs) are the target and effector cells of the lung injury process. Furthermore, mice model of sepsis caused by cecal ligation and puncture (CLP) and ALI cellular model induced by lipopolysaccharide (LPS) have been widely and successfully used (Meng *et al.,* 2019). During sepsis-induced ALI, some cytokines and mediators of inflammation can destroy the intercellular junctions, alter the cytoskeletal structure or destroy the cell monolayer, ultimately causing the gap in the endothelium, microvascular leakage and lung damage (Zhang *et al.,* 2012).

MicroRNAs (miRNAs) are a class of evolutionary highly conserved non-coding RNA molecules, which play vital roles in various physiological processes, including organ syndrome induced by sepsis, especially in ALI (Kingsley & Bhat 2017; Meng *et al.,* 2019). MiR-490 was proved to have important effects in many fields. Previous reports found that miR-490 plays an essential role in tumors (Liu *et al.,* 2015; Xu *et al.,* 2015). MiR-490 can also inhibit the apoptosis of cells in the injured spinal cord and reduce the expression of inflammatory factors by targeting MK2 (Zhang *et al.,* 2018). However, the effects of miR-490 on sepsis-induced lung injury have not been reported.

Multidrug resistance-associated protein 4 (MRP4), a member of ATP-binding cassette transporters, acts as an energy-dependent transporter for cyclic nucleotides. The occurrence of sepsis ALI is closely related to the reduction in intracellular cyclic adenosine phosphate (cAMP) levels (Schlegel & Waschke 2014). MRP4 is a transmembrane protein that actively transports cAMP in cells (Yaneff *et al.,* 2019). Therefore, MRP4 may have a profound regulatory effect on the dysfunction of vascular endothelial barrier in sepsis (Xia *et al.,* 2019).

In this study, mice sepsis-induced ALI and PMVECs models were established to study the effect of miR-490 on sepsis-induced ALI. The histopathology, injure score, Wet/Dry (W/D) ratio of lung tissue were assessed to verify that the model was successfully established. Furthermore, expression levels of inflammatory cytokines including TNF-α and IL-1β were also detected in ALI mice model. These results may provide new ideas for the treatment of infectious ALI.

MATERIAL AND METHODS

Animals

32 C57BL/6 mice (5 days of age, 20-22 g, 16 males and 16 females) were purchased from Beijing Wei Tong Li Hua Experimental Animal Technology Co., Ltd., (Beijing, China; license: SCXK (Beijing] 2012-0001). Mice were fed in specific pathogen-free (SPF) animal rooms at 22±2°C, with a humidity of 40% and 12/12-h light/dark cycle. Mice had free access to chow and tap water. All experiments were approved by the Ethics Committee of Ruian People's Hospital and performed following the Guide for the Care and Use of Laboratory Animals (1996).

CLP mice model and groups

After one-week acclimatization, mice were randomly divided into the Sham group (n=8) and CLP model group (n=24). Mice underwent CLP surgery to establish the CLP-induced sepsis mice model as previously described (Liu *et al.,* 2017; Sureshbabu *et al.,* 2018). Firstly, mice were anesthetized by 6% chloral hydrate (300 mg/kg, Sigma-Aldrich, St. Louis, MI, USA). Secondly, 2 cm long incision along the midline of the abdomen was performed to open the abdomen and expose the cecal. Thirdly, the cecal was ligated at 1/2 distance from its base of cecal, the cecal was punctured twice with an 18G needle, and a small amount of intestinal content was squeezed out. Finally, the bowel was sealed and the abdomen was closed using sterile sutures. After the operation, mice were injected subcutaneously with 0.9% saline (30 ml/kg) to be resuscitated. Sham group (n=8): mice received only laparotomy for cecal exploration without ligation and perforation. recorded.

Delivery of miR-490 *in vivo*

The miRNA reagents, miR-490 agomir and scrambled control (NC agomir) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and then mixed with linear polyethyleneimine (PEI) nanoparticles (Sigma-Aldrich, St. Louis, MO) as previously described (Morishita *et al.,* 2015). CLP mice were further randomly divided into 3 groups 7 days before the CLP operation. CLP group (n=8): mice underwent routine CLP surgery; CLP+NC agomir (n=8): mice were intravenously injected with a total of 200 μL mixture containing 5 nM NC agomir via the tail vein, and then underwent routine modeling surgery; CLP+miR-490 agomir (n=8): mice were intravenously injected with a total of 200 μL mixture containing 5 nM miR-490 agomir via the tail vein, and then underwent routine modeling surgery. The mice were sacrificed and lungs were collected for further analysis.

Histological analysis

The lungs specimens were embedded in paraffin, sectioned, and stained using hematoxylin and eosin (H&E). The histological staining was examined under a DP73 Digital microscope (Olympus Corp., Tokyo, Japan). The degree of lung injury was estimated in a blinded manner.

Wet/Dry (W/D) ratio of lung tissue

After isolation of the lung tissue, the surface moisture was absorbed, then the wet mass (W) was weighed. The tissue was dried at 70°C for 48 h, then the dry mass (D) was weighed, and the lung W/D ratio was calculated.

BALF sample collection and measurements

The airway was washed with 10 ml physiological saline 3 times: 4 ml, 4 ml and 3 ml. The recovered solution was collected and centrifuged at 1,500 rpm for 10 min at 4°C, then the supernatant was collected and stored at –20°C. The total protein concentration was calculated using the BCA protein assay kit (Beyotime Biotechnology, China) according to the instructions. After centrifugation, BALF was resuspended with 50 ml 0.9% normal saline, and the number of cells in BALF was counted with a cell counter. The neutrophil number was assessed by ELISA kit (Denley Dragon Wellscan MK 3, Thermo, Vantaa, Finland) according to the manufacturer's protocol. Macrophage number was assessed using M-CSF ELISA Kit according to the manufacturer's protocol (Shanghai Renjie Biotechnology Co., LTD, China). Each sample was analyzed in triplicate.

Cell culture, transfection, and grouping

MPVECs were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in Dulbecco's Modified Eagle Media (DMEM; Gibco, Invitrogen, Grand Island, NY, USA). The miR-490 mimics and control mimics (50 nM) were synthesized by GenePharm and transfected into MPVECs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 24 hours after transfection, cells were stimulated with 1 mg/mL lipopolysaccharide (LPS, Sigma-Aldrich); MPVECs were randomly divided into 7 groups. Ctrl group: MPVECs were cultured routinely; LPS group: MPVECs were stimulated with 1 mg/mL LPS (Sigma-Aldrich) only; LPS+NC agomir group: MPVECs were transfected with control mimics (50 nM) and then stimulated with 1 mg/mL LPS (Sigma-Aldrich); LPS+miR-490 agomir group: MPVECs were transfected with miR-490 agomir and then stimulated with 1 mg/mL LPS (Sigma-Aldrich); LPS+NC agomir+vehicle: MPVECs were transfected with control mimics (50 nM) and the empty virus control, then stimulated with 1 mg/mL LPS (Sigma-Aldrich); LPS+miR-490 agomir+vehicle group: MPVECs transfected with miR-490 agomir and empty virus control then stimulated with 1 mg/mL LPS (Sigma-Aldrich); LPS+miR-490 agomir+MRP4 group: MPVECs were transfected with miR-490 and recombinant adenovirus expressing MRP4, then stimulated with 1 mg/mL LPS (Sigma-Aldrich).

qRT-PCR

Total RNA was isolated from the lung tissues of mice and MPVECs by homogenization with Trizol (Invitrogen, Carlsbad, CA, USA), according to the

manufacturer's protocol. Total RNA was checked using a FOTODYNE gel imaging analysis system (Fotodyne, Inc., Hartland, WI, USA) to determine its quality and 28S/18S ratio. cDNA synthesis was performed using a cDNA Cycle Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The primer sequences of miR-490 and U6 were as follows: miR-490, F: 5'-ATCCAGTGCGTGTCGTG-3'; R: 5'-TGCTTAAGTGGTTCCATGTT-3'; U6, F-5'-TGCGGGTGCTCGCTTCGGCAGC-3'; R: 5'-CCAGTGCAGGGTCCGAGGT-3'. qPCR analysis was performed using MyiQ2 PCR thermocycling instrument (Bio-Rad, Shanghai, China). The expression levels of U6 were used to normalize the mRNA expression and fold change=$2^{-\Delta\Delta}$CT equation was used for calculations.

Western blot

Total protein was isolated from MPVECs and quantified using a protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Equal amounts of protein (20 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). Non-specific sites were blocked with 5% milk powder diluted in TBS with 0.05% Tween 20 (TBST). The membranes were incubated overnight at 4°C with rabbit anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Cleaved caspase-3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-MRP4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the membranes were washed repeatedly with TBST, they were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000 dilution; Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China). The blots were assessed by enhanced chemiluminescence. The expression levels of Pin1 and mTOR in esophageal epithelial tissue were quantified by densitometry using the Quantity One software (Bio-Rad, Hercules, CA, USA). The expression levels of Bcl-2, cleaved caspase-3, and MRP4 were normalized to GAPDH.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNF-α and IL-1β in mice BALF, lung tissue and MPVEC lysates were measured with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction.

Dual-luciferase reporter assay

The MRP4 3' -UTR fragment containing the possible binding site of miR-490 was amplified and cloned into the pmirGlo vector (GenePharm). MRP4-MUT vector was constructed using the mutant MRP4 3' -UTR. 293T cells were seeded into 96-well plates at a density of 5×10^3 cells per well. Then, 293T cells (ATCC; Manassas, VA) were co-transfected with miR-490 mimics (or NC mimics) and MRP4-WT (or MRP4-MUT) at 70–80% confluency. After 24-h incubation, pMIR-luciferase activity was detected by Dual-luciferase Reporter Assay System (Promega, USA) as described in the manufacturer's instructions.

Apoptosis assay

Annexin-V FITC kit (Beijing BaosaiBiotechnology) was used to assess MPVECs apoptosis. Briefly, after MPVECs were treated as mentioned previously the cells were washed with PBS buffer twice, then 1×10^6 /mL cell suspension was prepared with 1× Binding Buffer. Annexin-V and nucleic acid dye were added to 100 ul of the suspension, mixed gently and incubated for 15 min. The cells were washed with 1× Binding Buffer once and the supernatant was removed. Then 5 ul PI were added to the cells, mixed and incubated for 15 min. Finally, 400 ul of 1× Binding Buffer was added to the mixture, incubated for 1 h and the samples were analyzed by flow cytometry.

Statistical analysis

All data were presented as means ± standard deviations. The differences between the groups were assessed using one-way Analysis Of Variance (ANOVA) with the Student-Newman-Keuls (SNK) test for posthoc analysis. All data were analyzed using SPSS 17.0 (IBM, Armonk, NY, USA). $p<0.05$ was considered statistically significant.

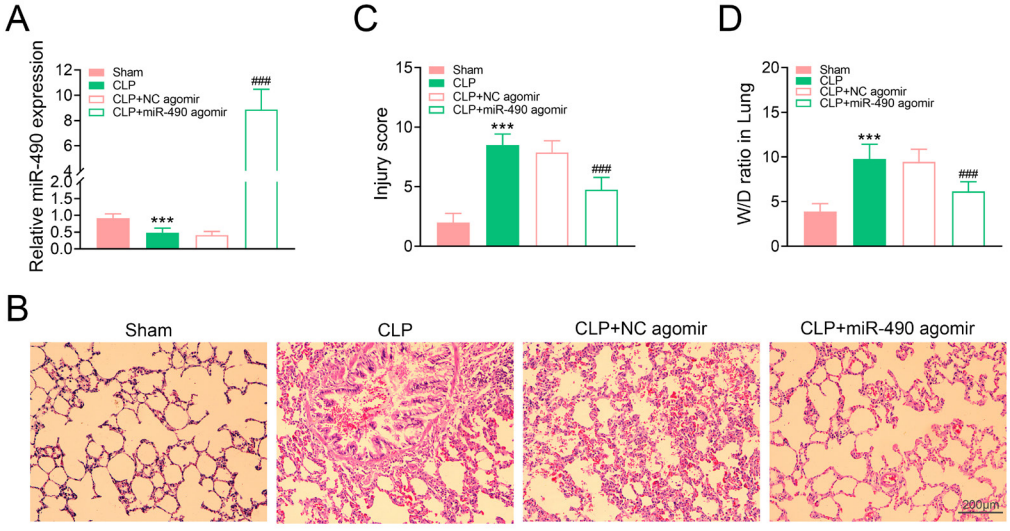

Figure 1. CLP downregulated miR-490 in mice and caused lung injury, and overexpression of miR-490 alleviated the lung injury (A); the levels of miR-490 in mice detected by qRT-PCR; (B) histopathological H&E staining of mice lung tissue; (C) the injury degree score of mice lung tissue; (D) the W/D ratio of mice lung tissue. ***$p<0.01$, compared to the sham group; ###$p<0.01$, compared to CLP +NC agomir group.

RESULTS

MiR-490 was downregulated in CLP-induced sepsis mice and the up-regulation of miR-490 alleviated sepsis-induced ALI

In order to study the role of miR-490 in ALI in sepsis mice, CLP was used to establish a mouse sepsis model. Compared to the sham group, miR-490 was significantly downregulated in CLP mice. However, the expression of miR-490 was significantly increased after the injection of miR-490 agomir when compared to that of NC agomir (Fig. 1A). As shown in Fig. 1B, compared to the sham group, the pulmonary tissues of mice in the sepsis group were significantly hyperemic, and alveolar collapse and alveolar wall thickening were present. However, these symptoms were relieved in comparison to the group injected with miR-490 agomir (Fig. 1B). Lung injury score was used to assess the extent of lung tissue damage, and overexpression of miR-490 significantly improved the histological damage caused by CLP surgery (Fig. 1C). Compared to the sham group, the W/D ratio of the lung tissue from mice in the sepsis group was increased significantly. However, overexpression of miR-490 significantly reduced the W/D ratio of the lung tissue from sepsis mice (Fig. 1D). Taken together, these results revealed that up-regulation of miR-490 reduced CLP-induced ALI in mice.

Overexpression of miR-490 decreased the expression of inflammatory cytokines in sepsis mice model

BALF and lung tissue were collected to examine the level of lung inflammatory cytokines in sepsis mice model. As shown in Fig. 2, compared to the sham group, the total protein concentration, cell count, neutrophils, macrophages, TNF-α and IL-1β were all increased in BALF from sepsis model mice. However, overexpression of miR-490 significantly decreased the total protein concentration, cell count, neutrophils, macrophages, TNF-α and IL-1β levels in BALF from sepsis model mice (Fig. 2A, 2B, 2C, 2D, 2E). Similarly, in the lung tissues from sepsis model mice, the expression of TNF-α and IL-1β was increased significantly compared to the sham mice, and overexpression of miR-490 significantly reduced the levels of TNF-α and IL-1β (Fig. 2F). To sum up, overexpression of miR-490 significantly decreased the production of pro-inflammatory cytokines in sepsis model mice.

Overexpression of miR-490 reduced LPS-induced inflammation and apoptosis in PMVECs

In order to study the effect of miR-490 on PMVECs, mice PMVECs were stimulated with LPS to establish a cell model of ALI, and cells were transfected with miR-490 agomir. qRT-PCR results showed that, compared to the control group, LPS stimulation significantly reduced the levels of miR-490. Besides, the transfection with miR-490 agomir significantly enhanced miR-490 expression (Fig. 3A). Flow cytometry data showed that LPS could promote the apoptosis of MPVECs, and miR-490 agomir transfection could effectively decrease the apoptotic rate of MPVECs (Fig. 3B). Further analysis revealed that overexpression of miR-490 increased the protein level of Bcl-2 and decreased the protein level of cleaved caspase-3 compared to the transfection with NC agomir (Fig. 3C). Overexpression of miR-490 significantly reduced the protein levels of TNF-α and IL-1β (Fig. 3D).

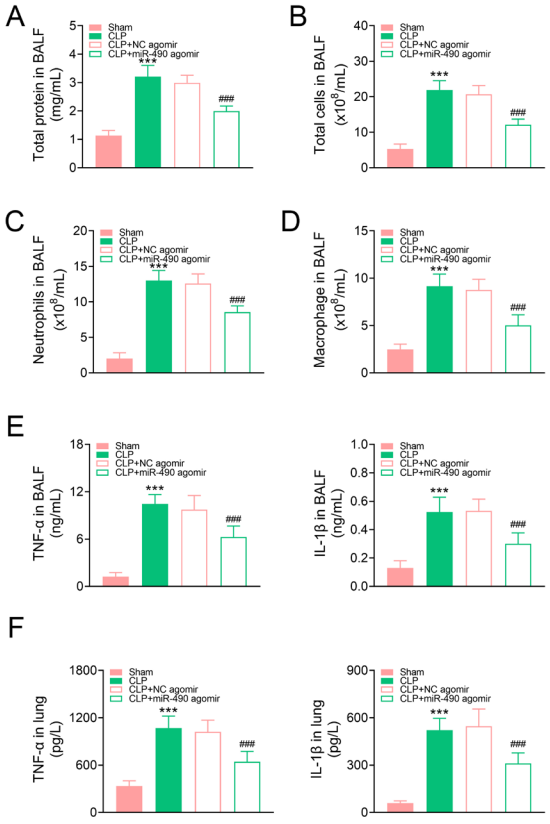

Figure 2. Overexpression of miR-490 decreased the expression of inflammatory cytokines in sepsis mice models.
(**A**) The total protein concentration calculated using the BCA protein assay kit; (**B**) the number of cells in BALF counted using a cell counter; (**C**) Neutrophils detected using ELISA kit; (**D**) Macrophages detected using M-CSF ELISA Kit; (**E**) Concentrations of TNF-α and IL-1β in mice BALF measured with an ELISA kit; (**F**) Concentration of IL-1β in mice BALF and lung tissue measured by an ELISA kit. ***$p<0.01$, compared to the sham group; ###$p<0.01$, compared to the CLP +NC agomir group.

In summary, overexpression of miR-490 reduced LPS-induced inflammation and apoptosis of PMVECs.

MRP4 was a potential downstream target of miR-490

To explore the potential mechanism of miR-490 action in ALI, we performed bioinformatics analysis using TargetScan (http://www.targetscan.org/vert_71/) to predict the downstream targets of miR-490. MRP4 was predicted as a direct target gene of miR-490, with putative binding sites (Fig. 4A). The dual-luciferase reporter gene experiment showed that in cells co-transfected with miR-490 mimics and MRP4-WT, the luciferase activity was significantly lower than that in case of transfection with miR-490 NC and MRP4-WT. However, there were no significant differences in the luciferase activity between cells co-transfected with MRP4-MUT+miR-490 and the MRP4-MUT+NC group (Fig. 4B). In addition, we measured the expression of MRP4 found that the expression level of MRP4 in LPS-treated cells and CLP group was higher than that in the corresponding control and sham group, respectively. However, the expression levels of MRP4 in LPS+miR-490 agomir group and CLP+miR-490 agomir group were significantly lower than that in LPS+NC agomir group and CLP+NC agomir group, respectively, indicating that miR-490 could negatively regulate the expression of MRP4 (Fig. 4C, 4D).

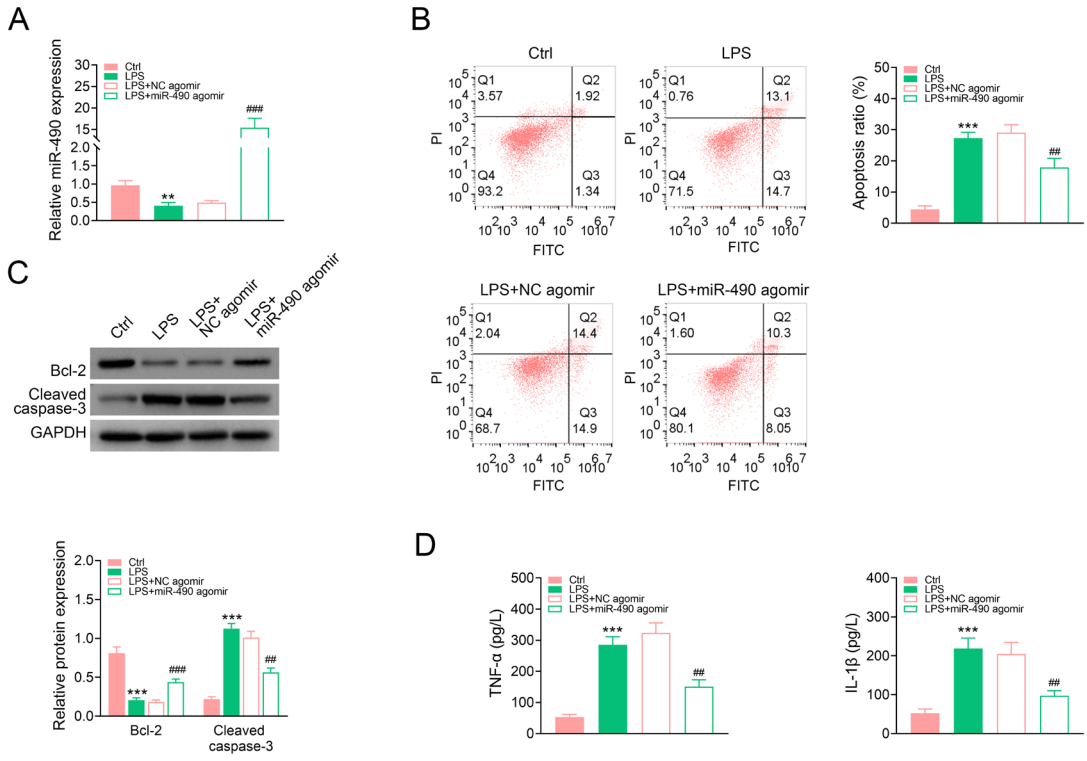

Figure 3. Overexpression of miR-490 reduced LPS-induced inflammation and apoptosis of PMVECs.
(**A**) Levels of miR-490 in PMVECs detected using qRT-PCR; (**B**) Apoptosis of MPVECs cells detected using flow cytometry; (**C**) The levels of Bcl-2 and cleaved caspase-3 detected using WB; (**D**) Concentrations of TNF-α and IL-1β in PMVECs measured with an ELISA kit. ***p*<0.05, ****p*<0.01, compared to the control group; ##*p*<0.05, ###P<0.01, compared to the LPS+NC agomir group.

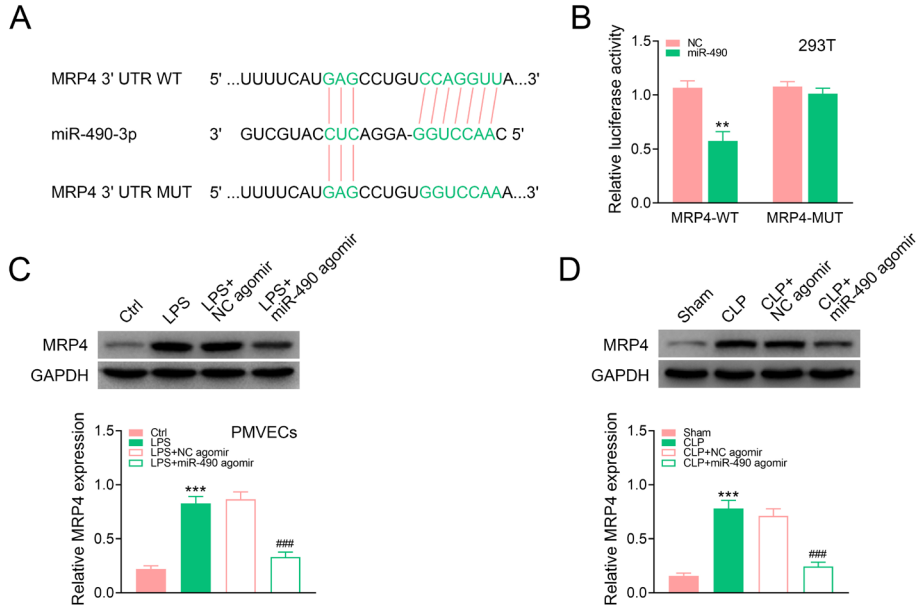

Figure 4. MRP4 was a potential downstream target of miR-490.
(**A**) Potential alignment of miR-490 binding sites in MRP4; (**B**) Luciferase activity measured in 293T cells; (**C**) The expression of MRP4 in transfected cells detected by WB; (**D**) The expression of MRP4 in lung tissues detected by WB. ***p*<0.05, compared to the NC; ****p*<0.01, compared to the control group; ###*p*<0.01, compared to the LPS+NC agomir group.

MiR-490 alleviates LPS-induced lung cell injury by regulating MRP4 expression

To demonstrate whether miR-490 can alleviate LPS-induced lung cell damage by regulating MRP4 expression, cells were transfected with recombinant adenovirus expressing MRP4. As shown in Fig. 5A, after the transfection the effects of miR-490 agomir on the expression levels of MRP4, Bcl-2 and cleaved caspase-3 were reversed. The transfection with recombinant adenovirus expressing MRP4 significantly increased the levels of TNF-α and IL-1β decreased by miR-490 agomir (Fig. 5B). Taken together, these results suggested that

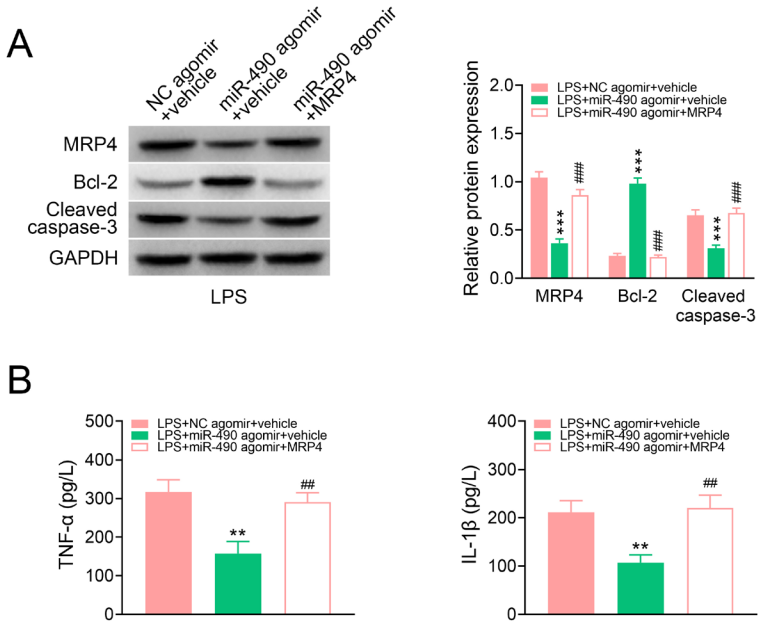

Figure 5. MiR-490 alleviated LPS-induced lung cell injury by regulating MRP4 expression.
(**A**) Levels of MRP4, Bcl-2 and cleaved caspase-3 detected using W (**B**) Concentrations of TNF-α and IL-1β in PMVECs measured using ELISA kits. ***p*<0.05, ****p*<0.01, compared to the LPS+NC agomir+vehicle group; ###*p*<0.01, ##*p*<0.05, compared to the LPS+miR-490 agomir+vehicle group.

miR-490 alleviated LPS-induced lung cell injury by regulating MRP4 expression.

DISCUSSION

Sepsis-induced ALI is a serious inflammatory disease of the lungs, accompanied by alveolar capillary inflammatory damage and inflammatory cytokines production (Miyashita *et al.,* 2016). At present, the mortality rate of sepsis-ALI and ARDS has reached 40%, which is one of the main reasons of death in ICUs (Singh *et al.,* 2014). Previous studies explored the pathogenesis and pathophysiology of sepsis-induced ALI in terms of genes and signaling pathways, but it is still not fully understood (Zhang *et al.,* 2018; Meng *et al.,* 2019). To provide new ideas for the treatment of sepsis-induced ALI, we thus studied the role and related mechanisms of miR-490 in sepsis-induced ALI. In the current study, we simultaneously established animal and cell models to investigate the role of miR-490 in ALI *in vitro* and *in vivo* and reveal its mechanism of action.

Firstly, we established a mice model of sepsis by CLP. Results showed that the lung injury score washigh, the histological examination revealed obvious congestion of the lungs, the collapse of the alveoli, thickening of the alveolar wall, and W/D results showed obvious pulmonary edema. These results were similar to previous studies, proving that a mouse model of sepsis was successfully established (Hamaguchi *et al.,* 2015; Deng *et al.,* 2018).

It is generally believed that the occurrence of ALI is related to the inflammatory cascade and neutrophil adhesion (Jia *et al.,* 2016). TNF-α and IL-1β are key cytokines in the inflammatory response in the body, and also important factors leading to lung injury in sepsis. Controlling excessive inflammatory response is the key to treat ALI (Park *et al.,* 2001; Lee *et al.,* 2018). In the present study, the number of neutrophils, macrophages, and the levels of TNF-α and IL-1β were increased significantly in ALI mice. Liu and others (Liu *et al.,* 2017) found that the level of TNF-α was markedly increased in the CLP group. Meng and others (Meng *et al.,* 2019) also found that IL-1β level was increased following CLP. Furthermore, the total protein concentration and cell number were increased significantly, indicating that the recruitment of inflammatory cells in alveoli damages the alveolar epithelium and pulmonary vascular endothelial cells, thus increasing the exudation of alveolar proteins and contributing to the occurrence of ALI. Overexpression of miR-490 was achieved through intravenous injection of miR-490 agomir. In the present study, overexpression of miR-490 significantly alleviate the increase in the neutrophils, macrophages, TNF-α and IL-1β levels, the total protein concentration, and cell numbers caused by CLP. These results indicated that overexpression of miR-490 could effectively reduce the recruitment of inflammatory cells and alveolar exudation, and thus relieve the symptoms of ALI. Ju and others (Ju *et al.,* 2018) found that microRNA-27a could inhibit inflammation and alleviate ALI in mice. Suo and others (Suo *et al.,* 2018) reported that miRNA-1246 could suppress ALI-induced inflammation in mice. Moreover, Xie and others (Xie *et al.,* 2018) found that miR-34b-5p could inhibit ALI inflammation.

Secondly, LPS was used to stimulate cells to establish cell models and investigate the effect of miR-490 *in vitro*. Similarly to animal experiments, the levels of TNF-α and IL-1β in LPS –treated PMVECs were increased, and overexpression of miR-490 could inhibit these effects. What is more, LPS decreased the levels of Bcl-2 and elevated the levels of cleaved caspase-3, which led to a high rate of apoptosis in PMVECs. However, overexpression of miR-490 effectively relieved the apoptosis of PMVECs. It is well-known that Bcl-2 and caspase-3 play an irreplaceable role in apoptosis (Liu *et al.,* 2020; Yuan & Zhang 2020). To sum up, this study indicated that the upregulation of miR-490 could affect the progression of ALI *in vivo and in vitro*. However, the mechanism of action remained unknown.

When studying the mechanism of miR-490 in ALI, we speculated that miR-490 might target specific molecules or regulate downstream genes to regulate inflammation and apoptosis-related pathways during ALI progression. MiR-490-3p could inhibit esophageal squamous cell carcinoma via targeting HMGA2 (Kang *et al.,* 2018). Overexpression of miR-490-5p inhibited the metastasis of hepatocellular carcinoma cells via targeting E2F2 and ECT2 (Fang *et al.,* 2018). Downregulation of miR-155 could inhibit lung apoptosis and inflammation via SIRT1 (Ju *et al.,* 2018). The downregulation of miR-1246 could decrease cells apoptosis, IL-1b, and neutrophil infiltration by targeting ACE2 (Fang *et al.,* 2017). It was also reported that miR-539-5p could alleviate ALI by targeting ROCK1 (Meng *et al.,* 2019). Here, the dual-luciferase assay indicated that miR-490 targeted MRP4. Moreover, overexpression of miR-490 inhibited the expression of MRP4 *in vitro* and *in vivo*.

The protective effect of MRP4 inhibition on ALI caused by sepsis may be exerted by reducing pulmonary vascular permeability and reducing inflammation. MRP4 can regulate the permeability of endothelial cells by regulating the downstream molecules - prostaglandins. It was reported that MRP4 inhibitor MK571 could reduce lung damage in rats with sepsis (Zheng *et al.,* 2016). In order to further investigate the mechanism of action, MRP4 was successfully overexpressed, and the results showed that overexpression of MRP4 could reverse the inhibitory effect of miR-490 overexpression on cell apoptosis and increase the levels of inflammatory factors. Xia, *et al.,* reported that inhibition of MRP4 could decrease serum inflammatory factors levels in BALF of septic mice, and the protective effect of MRP4 inhibition on sepsis-induced ALI might be associated with the elevation of cAMP levels (Xia *et al.,* 2019). cAMP is an important second messenger in cells, which can inhibit the damage dealt by inflammatory factors to the pulmonary vascular endothelium by increasing the intracellular cAMP level. The inhibition of MRP4 was also found to decrease inflammation significantly (Leite *et al.,* 2007). However, some limitations in the present study may exist. First, there are many cell types involved in ALI, such as endothelial cells, epithelial cells and inflammatory cells. However, the focus of this study was only on endothelial cells that proved to play an important role in ALI progression. Since MRP4 exists in various cell types and regulates various biological processes, it is necessary to further determine the regulatory effects of MRP4 on other cell types. Second, miR-490 may produce its biological effects by regulating other proteins, such as Golgi intermediate compartment protein 3 (ERGIC3). Besides, our study did not involve MRP4 silencing experiment, to prove the biological role of miR-490-MRP4 interaction.

In conclusion, miR-490 relieved the sepsis-induced ALI via downregulating the expression of MRP4, which indicates that miR-490 might act as a potential therapeutic target for sepsis-induced ALI.

Acknowledgments

Not applicable.

Funding

Not applicable.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

All experiments in this study were approved by the Ethics Committee of Ruian People's Hospital and performed following the Guide for the Care and Use of Laboratory Animals.

Statement of Informed Consent

Not applicable.

Authors' contributions

JL designed the study, supervised the data collection, analyzed the data, ZL interpreted the data and prepared the manuscript for publication, LL supervised the data collection, analyzed the data and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

REFERENCES

Amin Z,Rahmawati FN (2017) Recent insight into potential acute respiratory distress syndrome. *Saudi Med J* **38**: 344–349. https://doi.org/10.15537/smj.2017.4.15843

Aziz M, Ode Y, Zhou M, Ochani M, Holodick NE, Rothstein TL, Wang P (2018) B-1a cells protect mice from sepsis-induced acute lung injury. *Mol Med* **24**: 26. https://doi.org/10.1186/s10020-018-0029-2

Deng Q, Zhao T, Pan B, Dennahy IS, Duan X, Williams AM, Liu B, Lin N, Bhatti UF, Chen E, Alam HB, Li Y (2018) Protective effect of tubastatin A in CLP-induced lethal sepsis. *Inflammation* **41**: 2101–2109. https://doi.org/10.1007/s10753-018-0853-0

Fang Y, Gao F, Hao J, Liu Z (2017) microRNA-1246 mediates lipopolysaccharide-induced pulmonary endothelial cell apoptosis and acute lung injury by targeting angiotensin-converting enzyme 2. *Am J Transl Res* **9**: 1287–1296. PMID: 28386354

Fang ZQ, Li MC, Zhang YQ, Liu XG (2018) MiR-490-5p inhibits the metastasis of hepatocellular carcinoma by down-regulating E2F2 and ECT2. *J Cell Biochem* **119**: 8317–8324. https://doi.org/10.1002/jcb.26876

Guide for the Care and Use of Laboratory Animals (1996) Washington (DC). ISBN-10: 0-309-05377-3

Hamaguchi S, Akeda Y, Yamamoto N, Seki M, Yamamoto K, Oishi K, Tomono K (2015) Origin of Circulating Free DNA in Sepsis: Analysis of the CLP Mouse Model. *Mediators of Inflammation* **2015**: 614518–614518. https://doi.org/10.1155/2015/614518

Jia H, Sodhi CP, Yamaguchi Y, Lu P, Martin LY, Good M, Zhou Q, Sung J, Fulton WB, Nino DF, Prindle T Jr, Ozolek JA, Hackam DJ (2016) Pulmonary epithelial TLR4 activation leads to lung injury in neonatal necrotizing enterocolitis. *J Immunol* **197**: 859–871. https://doi.org/10.4049/jimmunol.1600618

Ju M, Liu B, He H, Gu Z, Liu Y, Su Y, Zhu D, Cang J, Luo Z (2018) MicroRNA-27a alleviates LPS-induced acute lung injury in mice via inhibiting in fl ammation and apoptosis through modulating TLR4/MyD88/NF-kappaB pathway. *Cell Cycle* **17**: 2001–2018. https://doi.org/10.1080/15384101.2018.1509635

Kang NN, Ge SL, Zhang RQ, Huang YL, Liu SD, Wu KM (2018) MiR-490-3p inhibited the proliferation and metastasis of esophageal squamous cell carcinoma by targeting HMGA2. *Eur Rev Med Pharmacol Sci* **22**: 8298–8305. https://doi.org/10.26355/eurrev_201812_16527

Kingsley SMK, Bhat BV (2017) Role of microRNAs in sepsis. *Inflamm Res* **66**: 553–569. https://doi.org/10.1007/s00011-017-1031-9

Lee JW, Chun W, Kwon OK, Park HA, Lim Y, Lee JH, Kim DY, Kim JH, Lee HK, Ryu HW, Oh SR, Ahn KS (2018) 3,4,5-Trihydroxycinnamic acid attenuates lipopolysaccharide (LPS)-induced acute lung injury via downregulating inflammatory molecules and upregulating HO-1/AMPK activation. *Int Immunopharmacol* **64**: 123–130. https://doi.org/10.1016/j.intimp.2018.08.015

Leite DF, Echevarria-Lima J, Ferreira SC, Calixto JB, Rumjanek VM (2007) ABCC transporter inhibition reduces zymosan-induced peritonitis. *J Leukoc Biol* **82**: 630–637. https://doi.org/10.1189/jlb.0107042

Liu C, Hu J, Mao Z, Kang H, Liu H, Fu W, Lv Y, Zhou F (2017) Acute kidney injury and inflammatory response of sepsis following

cecal ligation and puncture in d-galactose-induced aging rats. *Clin Interv Aging* **12**: 593–602. https://doi.org/10.2147/CIA.S132277

Liu W, Xu G, Liu H, Li T (2015) MicroRNA-490-3p regulates cell proliferation and apoptosis by targeting HMGA2 in osteosarcoma. *FEBS Lett* **589**: 3148–3153. https://doi.org/10.1016/j.febslet.2015.08.034

Liu Z, Zhang J, Zhang F, Chang Y (2020) Propofol post-conditioning lessens renal ischemia/reperfusion-induced acute lung injury associated with autophagy and apoptosis through MAPK signals in rats. *Gene* **741**: 144562. https://doi.org/10.1016/j.gene.2020.144562

Meng L, Cao H, Wan C, Jiang L (2019) MiR-539-5p alleviates sepsis-induced acute lung injury by targeting ROCK1. *Folia Histochem Cytobiol* **57**: 168–178. https://doi.org/10.5603/FHC.a2019.0019

Miyashita T, Ahmed AK, Nakanuma S, Okamoto K, Sakai S, Kinoshita J, Makino I, Nakamura K, Hayashi H, Oyama K, Tajima H, Takamura H, Ninomiya I, Fushida S, Harmon JW, Ohta T (2016) A three-phase approach for the early identification of acute lung injury induced by severe sepsis. *In Vivo* **30**: 341–349. PMID: 27381595

Morishita Y, Imai T, Yoshizawa H, Watanabe M, Ishibashi K, Muto S, Nagata D (2015) Delivery of microRNA-146a with polyethylenimine nanoparticles inhibits renal fibrosis *in vivo*. *Int J Nanomedicine* **10**: 3475–3488. https://doi.org/10.2147/IJN.S82587

Ning Q, Liu Z, Wang X, Zhang R, Zhang J, Yang M, Sun H, Han F, Zhao W, Zhang X (2017) Neurodegenerative changes and neuroapoptosis induced by systemic lipopolysaccharide administration are reversed by dexmedetomidine treatment in mice. *Neurol Res* **39**: 357–366. https://doi.org/10.1080/01616412.2017.1281197

Park WY, Goodman RB, Steinberg KP, Ruzinski JT, Radella F 2nd, Park DR, Pugin J, Skerrett SJ, Hudson LD, Martin TR (2001) Cytokine balance in the lungs of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* **164**: 1896–1903. https://doi.org/10.1164/ajrccm.164.10.2104013

Sagy M, Al-Qaqaa Y, Kim P (2013) Definitions and pathophysiology of sepsis. *Curr Probl Pediatr Adolesc Health Care* **43**: 260–263. https://doi.org/10.1016/j.cppeds.2013.10.001

Schlegel N, Waschke J (2014) cAMP with other signaling cues converges on Rac1 to stabilize the endothelial barrier- a signaling pathway compromised in inflammation. *Cell Tissue Res* **355**: 587–596. https://doi.org/10.1007/s00441-013-1755-y

Tg S, Jo S, Hwang S, Jo I, Lee T, Yoon H, Cha W, Sim M (2018) Ketamine use for endotracheal intubation in severe sepsis and septic shock. *Signa vitae* **14**: 24–30. https://doi.org/10.22514/SV142.102018.3

Singh G, Gladdy G, Chandy TT, Sen N (2014) Incidence and outcome of acute lung injury and acute respiratory distress syndrome in the surgical intensive care unit. *Indian J Crit Care Med* **18**: 659–665. https://doi.org/10.4103/0972-5229.142175

Suo T, Chen GZ, Huang Y, Zhao KC, Wang T, Hu K (2018) miRNA-1246 suppresses acute lung injury-induced inflammation and apoptosis *via* the NF-kappaB and Wnt/beta-catenin signal pathways. *Biomed Pharmacother* **108**: 783–791. https://doi.org/10.1016/j.biopha.2018.09.046

Sureshbabu A, Patino E, Ma KC, Laursen K, Finkelsztein EJ, Akchurin O, Muthukumar T, Ryter SW, Gudas L, Choi AMK, Choi ME (2018) RIPK3 promotes sepsis-induced acute kidney injury via mitochondrial dysfunction. *JCI Insight* **3**: https://doi.org/10.1172/jci.insight.98411

Xia W, Zhang H, Pan Z, Li G, Zhou Q, Hu D, Liu Y (2019) Inhibition of MRP4 alleviates sepsis-induced acute lung injury in rats. *Int Immunopharmacol* **72**: 211–217. https://doi.org/10.1016/j.intimp.2019.04.009

Xie W, Lu Q, Wang K, Lu J, Gu X, Zhu D, Liu F, Guo Z (2018) miR-34b-5p inhibition attenuates lung inflammation and apoptosis in an LPS-induced acute lung injury mouse model by targeting progranulin. *J Cell Physiol* **233**: 6615–6631. https://doi.org/10.1002/jcp.26274

Xu X, Chen R, Li Z, Huang N, Wu X, Li S, Li Y, Wu S (2015) MicroRNA-490-3p inhibits colorectal cancer metastasis by targeting TGFbetaR1. *BMC Cancer* **15**: 1023. https://doi.org/10.1186/s12885-015-2032-0

Yaneff A, Sahores A, Gómez N, Carozzo A, Shayo C, Davio C (2019) MRP4/ABCC4 As a new therapeutic target: meta-analysis to determine cAMP BINDING SITES AS A TOOL FOR DRUG DEsign. *Curr Med Chem* **26**: 1270–1307. https://doi.org/10.2174/0929867325666171229133259

Yuan J, Zhang Y (2020) Sevoflurane reduces inflammatory factor expression, increases viability and inhibits apoptosis of lung cells in acute lung injury by microRNA-34a-3p upregulation and STAT1 downregulation. *Chem Biol Interact* **322**: 109027. https://doi.org/10.1016/j.cbi.2020.109027

Zhang LN, Wang XT, Ai YH, Guo QL, Huang L, Liu ZY, Yao B (2012) Epidemiological features and risk factors of sepsis-associated encephalopathy in intensive care unit patients: 2008-2011. *Chin Med J (Engl)* **125**: 828–831. PMID: 22490582

Zhang ZT, Zhang XG, Yu HY, Xu ZT (2018) MicroRNA-490-3p inhibits inflammation and apoptosis induced by spinal cord injury via targeting MK2. *Eur Rev Med Pharmacol Sci* **22**: 8849–8856. https://doi.org/10.26355/eurrev_201812_16653

Zheng Y, Xia W, Zhou Q, Su B, Zhang H (2016) Protective effect of multidrug resistant associated protein 4 inhibitor on rats with sepsis-induced acute lung injury. *Chin Crit Care Med* **028**: 504–507