

# Mechanisms underlying the improvement of preeclampsia through salvianolic acid B-regulated miRNA-155/CXCR4

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## Abstract

**Introduction:** The aim of this study was to evaluate the effects and mechanisms of salvianolic acid B (Sal B) in preeclampsia treatment by *in vivo* and *in vitro* study.

**Material and methods:** Rats were randomly divided into 5 groups. In order to establish the model of preeclampsia, endotoxin was administered to the rats in the Sal B intervention and model groups. The systolic blood pressure (SBP) of the tail artery and urine protein concentration were observed at different points, the miRNA-155 and CXCR4 gene expression levels by RT-PCR and the CXCR4 and p-AKT protein expression by WB assay. Using HTR8/SVneo to explain the mechanisms; evaluating the miRNA-155 and CXCR4 mRNA expression by RT-PCR assay, measuring the cell invasion and migration by transwell and wound healing assay in different groups; evaluating the CXCR4 and p-AKT protein expression by WB assay and p-AKT nucleation volume by cellular immunofluorescence were evaluated.

**Results:** Compared with the normal group, the systolic blood pressure and urine protein were significantly increased in the model group ( $p < 0.05$ ), serum NO concentration was significantly down-regulated (all  $p < 0.05$ ), CXCR4 and miRNA-155 mRNA expression was significantly different and CXCR4 and p-AKT protein expression was significantly suppressed (all  $p < 0.05$ ). With Sal B supplement, the SBP, urine protein and NO concentration were significantly improved with dose-dependent (all  $p < 0.05$ ). In the cell experiment, the cell invasion and migration ability were significantly improved with Sal B supplement (both  $p < 0.05$ ). However, with miRNA-155 transfection, the cell invasion and migration ability were suppressed with Sal B treatment (both  $p < 0.05$ ).

**Conclusions:** Sal B improved preeclampsia via regulation of miRNA-155/CXCR4 in the *in vitro* and *vivo* study.

**Key words:** Sal B, preeclampsia, HTR8/SVneo cell, miRNA-155, CXCR4, p-AKT, *Salvia miltiorrhiza*.

## Introduction

Preeclampsia (PE) is a type of hypertension specific to human pregnancy, with an incidence of 2% to 7%. PE is primarily clinically manifested as an increase in urinary protein and blood pressure after 20 weeks of gestation [1], and represents one of the main etiologies for maternal and perinatal morbidity and mortality. The high-risk factors for

PE include primiparity age over 40 years, obesity, diabetes mellitus, assisted reproductive conception, a family history of PE, multiple pregnancies, a previous pregnancy of PE and chronic hypertension [2]. To date, the mechanisms of pathogenesis underlying PE remain unclear. MicroRNAs (miRNAs) are a group of single-stranded non-coding small RNA molecules that are 18–23 nucleotides in length. Accumulating studies in recent years have shown that some miRNAs are abnormally expressed when PE occurs; one of them is miR-155 [3–5]. Abnormal expression levels of miRNA-155 may be closely associated with the development of such dysfunctions as trophoblast invasion and migration [6], whereas the specific molecular mechanisms still warrant more explorations and studies. As a G protein-coupled receptor, CXCR4 is expressed in the villus tissue throughout pregnancy. After binding to its ligand, CXCR4 can activate the downstream PI3K/AKT signaling pathway to affect the expression levels of genes within cells, thereby regulating the invasion and migration of the trophoblasts within the human villus tissues in early pregnancy [7, 8].

Salvianolic acid B (Sal B) is an aqueous bioactive component in *Salvia miltiorrhiza*, and a polyphenolic compound highly abundant within this plant. Sal B has the molecular formula of C<sub>36</sub>H<sub>30</sub>O<sub>16</sub>, an important water-soluble active ingredient in *Salvia miltiorrhiza*, and possesses most of the biological activity of *Salvia miltiorrhiza* [9]. Studies have shown that Sal B has been extensively used in various diseases, such as angina pectoris, pulmonary fibrosis and malignancies [10–12]. Sal B is most commonly used for treatment of cardiovascular disease, such as myocardial infarction and heart failure [9, 13, 14]; however, the roles of Sal B in improving PE and related mechanisms underlying it remain elusive.

Herein, we for the first time explored the efficacy of Sal B in PE through *in vivo* experiments, and initially investigated the roles of miRNA-155/CXCR4 in it; then, we validated the relevant mechanisms of action through cell experiments. By doing so, we investigated the mechanisms of action of Sal B in improving PE, and provided a solid experimental basis for the clinical treatment of PE.

## Material and methods

### Materials

Sal B was purchased from MCE (MedChemExpress, USA), and endotoxin from Sigma, USA, with a purity of 99.9%; the NO kit was a product from Shanghai Yanjing Biotechnology Co., Ltd., early pregnancy human extravillous trophoblast HTR8/SVneo from the Cell bank, Chinese Academy of Science, and fetal bovine serum and RPMI1640 me-

dium from Gibco, USA. miR-155 mimics, negative control (NC), miRNA-155 inhibitor and miRNA-155 inhibitor negative control (miRNA-155 inhibitor NC) were designed and synthesized by Shanghai Gene Pharma Co., Ltd., China. The sequences of miRNA-155 mimics (5' to 3') were: sense: UUAUGC-CAAUCGUGAUAGGGGU; antisense: CCCUAUCAC-GAUUAGCAUUAUU; the sequence of miRNA-155 inhibitor (5' to 3') was ACCCCUAUCACGUAUAGCAUUA; the sequences of NC (5' to 3') were: sense: UUCUCCGAACGUGUCACGUTT; antisense: ACGUGACACGUUCGGAGAATT; and the sequence of miRNA-155 inhibitor NC (5' to 3') was CAGUA-CUUUUGUGUAGUACAA. The following materials were used: RPMI1640 medium (Hyclone), Opti-MEM medium (Gibco), fetal bovine serum (FBS, Hangzhou Evergreen), trypsin (Geneview), transfection reagent Lipofectamine 2000 and Trizol (Invitrogen), RNA reverse transcription kit, SYBR green fluorescence Dye (Toyobo), Real-time PCR System, Transwell Chamber (BD Biosciences), protein concentration assay kit (Beijing CW Century Co., Ltd.), protein marker molecular marker (Geneview), rabbit anti-human CXCR4 monoclonal antibody, rabbit anti-human p-AKT monoclonal antibody, and rat anti-human  $\beta$ -actin monoclonal antibody (Abcam).

## Experimental methods

### Establishment and grouping of PE models

SD rats of clean grade with a body weight of 200–250 g were fed in a standard clean environment. The experiment was conducted in strict accordance with the National Institutes of Health (NIH) guidelines for feeding and use of laboratory animals. The male and female rats were housed in separate cages at a ratio of 2 : 1, with the finding of a vaginal plug as the 1<sup>st</sup> day of pregnancy. A total of 60 female rats were randomized to 5 groups, with 12 rats in each group. In addition to the normal group, the rats in the drug groups (low-, medium-, and high-dose groups) and the model control group were intravenously instilled with endotoxin 1.0  $\mu$ g/(kg·d) at 2 ml/h at the 14<sup>th</sup> day of pregnancy for 4 days, and the rats in the blank control group were given normal saline of an equal dose. The conditions for H/R were hypoxia at 37°C with 2% O<sub>2</sub> and 5% CO<sub>2</sub> for 2 h, and reoxygenation at 37°C with 20% O<sub>2</sub> and 5% CO<sub>2</sub> for 6 h [15].

### Methods of administration

From the 14<sup>th</sup> to the 21<sup>st</sup> day of pregnancy, the rats in the low-, medium- and high-dose drug groups were intraperitoneally injected with different concentrations of Sal B injection (20.0, 40.0, and 80.0 mg/kg), and the rats in the blank control and model control groups were administered nor-

mal saline of an equal dose until the 21<sup>st</sup> day of pregnancy.

#### Indicators for detection

The blood pressure of the tail artery, the content of 24 h urine protein and the concentration of plasma NO were monitored on the 19<sup>th</sup> and 21<sup>st</sup> days of pregnancy, respectively. 1) Test of blood pressure of the tail artery: the blood pressure of the rats' tail artery was measured by means of the multi-channel non-invasive pressure measurement system. The rat was fixed in an incubator at 30°C for about 10 min, and the probe was fixed. The blood flow was blocked by the pressure increased by the tail compression sleeve until the blood flow of the tail artery had been blocked and the pulse wave had disappeared. After 3 to 5 s, the software was turned on and the external pressure was gradually decreased until the arterial pulsation appeared, at which time the blood pressure was recorded as systolic blood pressure of the experimental animal. Each rat was measured repeatedly 6 times at an interval of 2 min, with the mean value used. 2) Content of 24 h urine protein: the 24 h urine of the rat was collected, and the concentration of urine protein was determined through colorimetry. 3) Concentration of the plasma NO: 5 ml of blood from the abdominal aorta was collected and centrifuged, with the supernatant taken for use. The absorbance value of the supernatant was measured using the nitrate reductase method and converted to the concentration of NO.

#### HE staining

The placental tissues were removed, partially fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned (4 µm). The placental tissues underwent HE staining for pathological examination.

#### Total RNA extraction, reverse transcription and amplification

The placental tissues or cells were taken out from the rats and subjected to extraction of total RNA using the Trizol method, and the concentrations and purity of RNA in all groups were determined. 2 µg of RNA was reverse transcribed into cDNA, with the volume of the reverse transcription system being 20 µl. Amplification was performed after reverse transcription, with 3 replicate wells set in each group. Primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd., and the primer sequences were as follows: CXCR-4 F: 5'-ACTACACCGAGGAAATGGGCT-3'; R: 5'-CCCACAATGCCAGTTAAGAAGA-3'; β-actin F: 5'-GGGAAATCGTGCGTGACATTAAGG-3'; R: 5'-CAG-

GAAGGAAGGCTGGAAGAGTG-3'. miRNA-155 F: 5'-TTAATGCTAATCGTGACT-3'; R: 5'-ACCTGAGAGTAGACCAGA-3'. U6 F: 5'-CTCGCTTCGGCAGCAC-3'; R: 5'-AACGCTTACGAATTTGCGT-3'. The amplification procedure was pre-denaturation at 95°C for 10 min; 95°C for 15 s; 60°C for 1 min (40 cycles in total); prolongation at 72°C for 10 min. The relative expression levels of target genes were used as the criterion, and the relative expression levels of CXCR4 and miRNA-155 mRNA were calculated using  $2^{-\Delta\Delta Ct}$ .

#### Cell culture, grouping and treatment

The RPMI1640 cell culture medium containing 10% fetal bovine serum was added to the HTR8/SVneo cells and cultured in a constant-temperature incubator at 37°C with 5% CO<sub>2</sub> and 95% saturated humidity. The cells were divided into the NC, H/R, BL, miRNA-155 mimics, miRNA-NC, inhibitor NC, miRNA-155 inhibitor, Sal B and Sal B + miRNA-155 groups.

#### Western blot

The fresh placenta tissues or cells from each group were placed, washed with pre-cooled PBS 3 times and then 50 µl of protein lysate (containing protease inhibitor and phosphatase inhibitor at 1 : 100) added to make the protein solution, whose concentration was determined using the BCA method. Protein loading was 80 µg, and 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) was prepared by means of electrophoresis for separation of proteins, followed by membrane transfer. CXCR4 was blocked with 5% skim milk powder, and p-AKT with 5% BSA at room temperature for 2 h, after which the blocking solution was discarded. Primary antibody CXCR4 (diluted at 1 : 500), p-AKT (diluted at 1 : 5 000) and internal reference β-actin (diluted at 15,000) antibody were added, incubated at 4°C overnight, secondary antibody (diluted at 1 : 1 : 1000) was added, and it was incubated at room temperature for 2 h, protected from light. The ODYSSEY Clx detection and imaging system was used for scanning and photographing.

#### Transwell cell invasion test

Prior to seeding of cells, it was necessary to equilibrate the transwell chamber and the 24-well plate: 100 µl of serum-free medium and 600 µl of FBS were added to the upper and lower chamber, respectively, and placed at 37°C with 5% CO<sub>2</sub> overnight. The cells in all the groups transfected and starved for 24 h were collected, counted, inoculated into the upper chamber at  $2 \times 10^4$  cells/well, and cultured at 37°C with 5% CO<sub>2</sub> for 36 h. Then, the cells in the upper chamber were erased, and

those in the lower chamber were fixed in methanol, and photographed after Giemsa staining. A total of 5 fields of view were used under high power microscope for counting, with the mean value used. The procedure for each group was repeated in triplicate.

### Wound healing experiment

The cells underwent the scratch test at 24 h after transfection. After the medium in the six-well plate had been replaced with serum-free medium for 12 h starvation of cells, a “hyphen”-shaped scratch was created with a 200  $\mu$ l pipette tip along the plate to form a central blank area. The tip was washed with PBS once, and the scratch area was photographed (0 h) under a microscope. Another 24 h culture was performed, and observation and photography (24 h) were done. The relative migration distance of cells was analyzed using Image J software. The experiment was repeated in triplicate, with the mean value used.

### Cellular immunofluorescence

After the cells in each group were treated for 48 h, the medium was discarded. The cells were washed with pre-cooled PBS, fixed in 2 ml of 4% paraformaldehyde for 20 min, permeabilized and blocked with 1% polyethylene glycol octylphenyl ether, to which rabbit anti-human p-AKT monoclonal antibody (1 : 100) was added, and incubated in a wet box at 4°C overnight. To the cells were then added FITC-labeled goat anti-rabbit fluorescent secondary antibody (diluted at 1 : 50, American Arthox, USA) dropwise, and incubated at 37°C for 1 h. The nuclei were stained with propidium iodide (PI) and the sections were sealed with glycerin. The colored locations and the intensity of fluorescence were observed under a laser confocal microscope.

### Statistical analysis

Statistical analysis was performed with the SPSS 20.0 statistical software. The experimental data in this study were represented by mean  $\pm$  SD. One-way analysis of variance was adopted for inter-group comparisons, and the SNK-q test for multiple inter-group comparisons. The test level was  $\alpha = 0.05$ .

## Results

### Histopathological effects of Sal B on placenta

Trophoblastic nodules and fibrinoid necrosis were rare in the placenta in the NC group, capillaries were abundant in number, and vascular syncytium cell membranes were well formed. Marked

nucleus vacuolarization of the syncytiotrophoblast cells was seen in the placental tissue in the model group, together with a large number of syncytial nodules, fibrinoid necrosis, and a decreased number of microvessels in the villi. After the administration of Sal B, occasional spiral arteries and some syncytial nodules were seen in the Sal B group, and constantly improved with the increase in the concentration of Sal B. See Figure 1 for details.

### Effects of Sal B on systolic blood pressure and urine protein concentration in PE rats

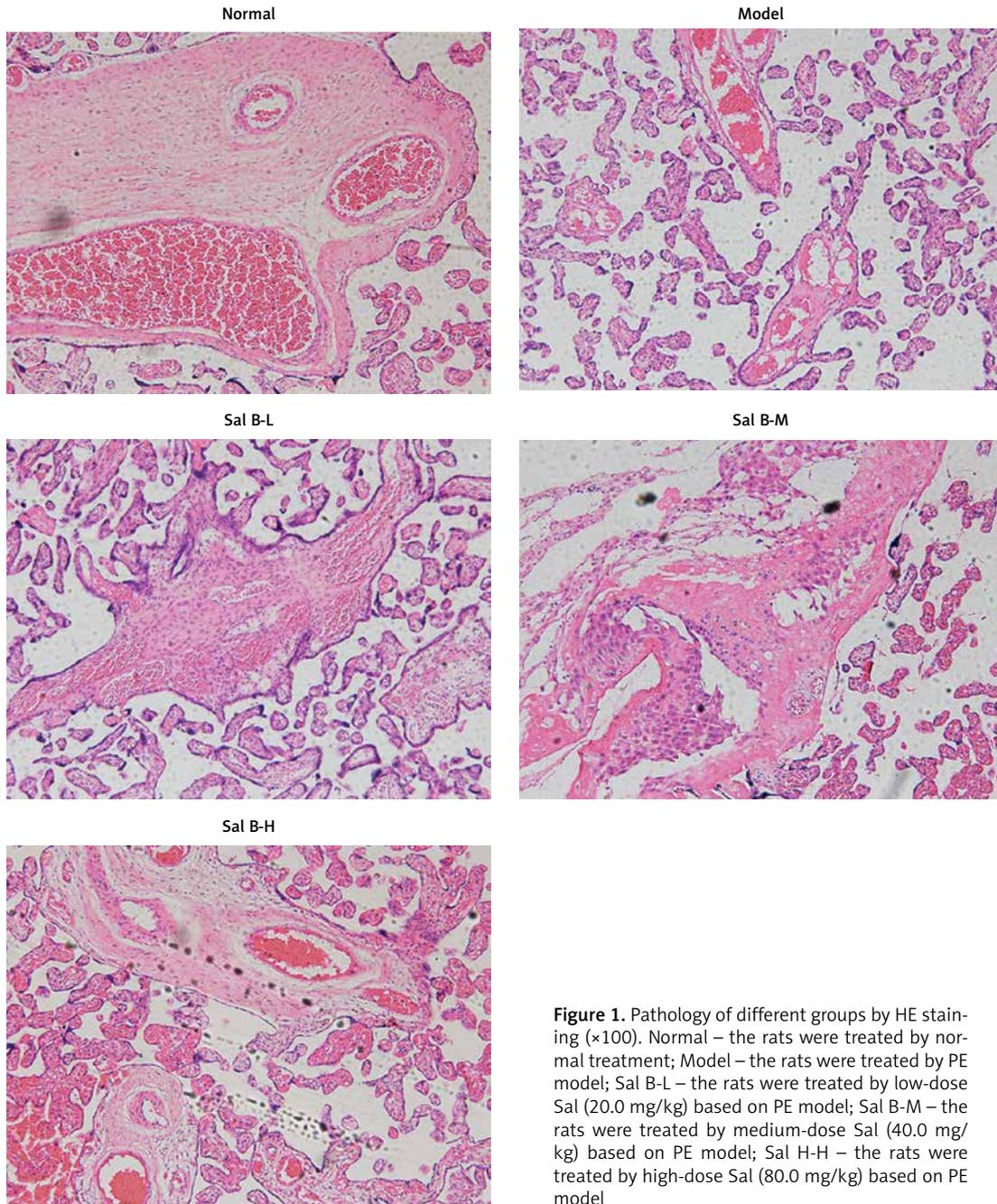
After induction with endotoxin drugs (D19), the systolic blood pressure and urine protein of rats in the model group and other drug groups were significantly higher than in the blank control group ( $p < 0.05$ ), suggestive of the successful establishment of the model. Model establishment between the low-, medium- and high-dose drug groups and the model group was comparable. At 4 days after the intervention of the various drug groups with the different concentrations of Sal B, the systolic blood pressure in each drug group was significantly lower than in the model group ( $p < 0.05$ ), and with the increase in the drug dose, the decrease in the systolic blood pressure was more obvious and the systolic blood pressure in the high-dose group was significantly lower than in the low-dose group ( $p < 0.05$ ). The concentration of urine protein in various drug groups was lower than in the model group, with the difference being statistically significant ( $p < 0.05$ ). The content of urine protein also showed a dose-dependent manner in different drug groups. After drug intervention, the concentrations of plasma NO in rats in various drug groups were generally higher than those prior to drug intervention, and significantly higher than in the model group ( $p < 0.05$ , Table I).

### Effects of Sal B on placental weight, body weight and body length of newborn rats

The placental weight, body weight and body length of the newborn rats in the model group were significantly lower than in the blank control group ( $p < 0.05$ ). The placental weight, body weight and body length of the newborn rats in the medium- and high-dose groups were significantly higher than in the model group ( $p < 0.05$ ). The placental weight, body weight and body length of the newborn rats in the low-dose group were higher than in the model control group, but the difference was not statistically significant (Table II).

### Effects of Sal B on miRNA-155, CXCR4 and p-AKT in the placenta of PE rats

RT-PCR quantitative analysis showed that compared with the normal group, the expression of



**Figure 1.** Pathology of different groups by HE staining (×100). Normal – the rats were treated by normal treatment; Model – the rats were treated by PE model; Sal B-L – the rats were treated by low-dose Sal (20.0 mg/kg) based on PE model; Sal B-M – the rats were treated by medium-dose Sal (40.0 mg/kg) based on PE model; Sal H-H – the rats were treated by high-dose Sal (80.0 mg/kg) based on PE model

**Table I.** Comparison of systolic blood pressure and urine protein concentration in preeclampsia rats of different points

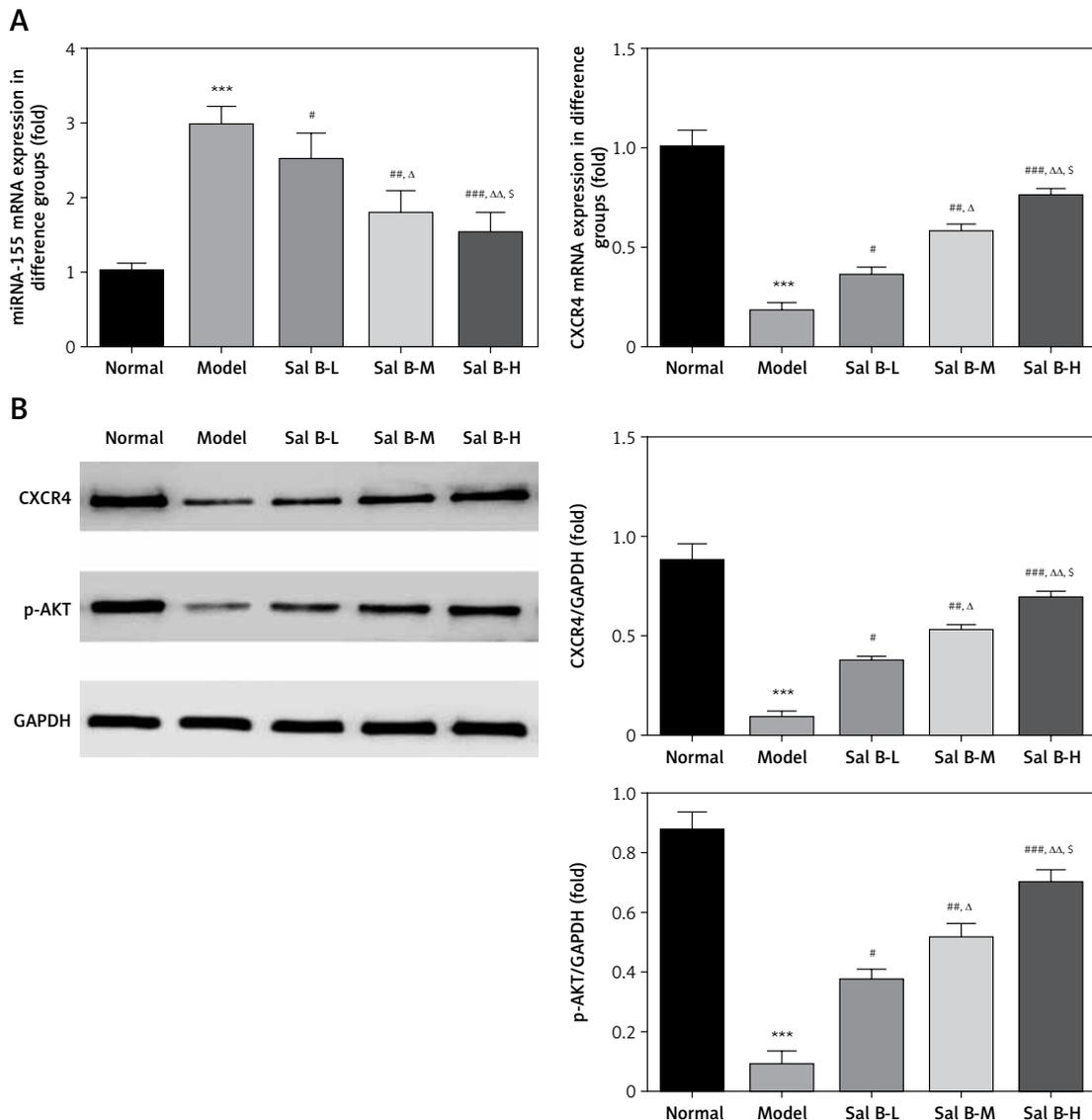
Group	N	SBP [mm Hg]		24 h proteinuria [mg/l]		NO [mmol/l]	
		D19	D21	D19	D21	D19	D21
Normal	12	113.7 ±4.2	115.8 ±3.5	142.8 ±22.6	140.5 ±22.5	154.8 ±28.8	153.4 ±33.5
Model	12	139.6 ±3.8*	138.5 ±4.2*	351.6 ±42.9*	360.5 ±50.9*	52.8 ±9.4*	54.6 ±8.8*
Sal B-L (20.0 mg/kg)	12	140.2 ±4.1*	128.1 ±3.8* <sup>Δ#</sup>	361.5 ±52.9*	208.5 ±35.9*	62.8 ±9.8*	72.8 ±10.8* <sup>Δ#</sup>
Sal B-M (40.0 mg/kg)	12	139.7 ±4.0*	125.2 ±4.0 <sup>Δ</sup>	355.8 ±48.5*	175.4 ±9.5*	58.4 ±8.9*	97.6 ±15.7* <sup>Δ#</sup>
Sal B-H (80.0 mg/kg)	12	139.5 ±4.8*	116.4 ±4.5 <sup>Δ</sup>	359.8 ±55.4*	153.7 ±10.5*	57.5 ±8.7*	130.5 ±20.6* <sup>Δ#</sup>

\*P < 0.05, compared with normal group; <sup>Δ</sup>p < 0.05, compared with model group; <sup>#</sup>p < 0.05, compared with Sal B-H group.

**Table II.** Placental weight, neonatal rats' weight and neonatal rats' body length in different groups

Group	N	Placental weight [g]	Neonatal rats' weight [g]	Neonatal rats' body length [cm]
Normal	12	0.73 ±0.13	5.24 ±0.55	3.89 ±0.35
Model	12	0.48 ±0.12	4.36 ±0.41*	3.12 ±0.35
Sal B-L (20.0 mg/kg)	12	0.51 ±0.14**	4.56 ±0.49**	3.24 ±0.40**
Sal B-M (40.0 mg/kg)	12	0.61 ±0.13* <sup>Δ</sup>	4.78 ±0.60* <sup>Δ</sup>	3.30 ±0.41 <sup>Δ</sup>
Sal B-H (80.0 mg/kg)	12	0.66 ±0.15 <sup>Δ</sup>	5.00 ±0.54* <sup>Δ</sup>	3.62 ±0.35 <sup>Δ</sup>

\*P < 0.05, compared with normal group; <sup>Δ</sup>p < 0.05, compared with model group; \*\*p < 0.05, compared with Sal B-H group.



**Figure 2.** miRNA-155 and CXCR4 mRNA by RT-PCR and CXCR4 and p-AKT protein expression by WB assay. Normal – the rats were treated by normal treatment; Model – the rats were treated by PE model; Sal B-L – the rats were treated by low-dose Sal (20.0 mg/kg) based on PE model; Sal B-M – the rats were treated by medium-dose Sal (40.0 mg/kg) based on PE model; Sal B-H – the rats were treated by high-dose Sal (80.0 mg/kg) based on PE model. **A** – miRNA-155 and CXCR4 mRNA expression by RT-PCR assay. **B** – CXCR4 and p-AKT protein expression by WB assay

\*\*\*P < 0.001, compared with normal group; #p < 0.05, ##p < 0.01, ###p < 0.001, compared with model group; <sup>Δ</sup>p < 0.05, <sup>ΔΔ</sup>p < 0.01, compared with Sal B-L group, <sup>S</sup>p < 0.05, compared with Sal B-M group.

the miRNA-155 gene was significantly increased while that of the CXCR4 gene was significantly decreased ( $p < 0.01$ , Figure 2 A) in the model group. Compared with the model group, the expression of the miRNA-155 gene was significantly inhibited while that of the CXCR4 gene was significantly elevated ( $p < 0.01$ , Figure 2 A) in the drug groups, with significant differences seen between the drug groups ( $p < 0.05$ , Figure 2 A). Semi-quantitative WB assay showed that compared with the normal group, the expression levels of the CXCR4 and p-AKT proteins in the model group were significantly lower ( $p < 0.01$ , Figure 2 B); compared with the model group, the expression levels of the CXCR4 and p-AKT proteins in the drug groups were significantly higher ( $p < 0.01$ , Figure 2 B), with significant differences seen between the drug groups ( $p < 0.05$ , Figure 2 B).

### Expression of miRNA-155 and CXCR4 genes in cells in various groups

Compared with the NC group, the expression of the miRNA-155 gene in H/R, BL, miRNA-155 mimics, miRNA-NC and inhibitor NC groups was significantly higher while the expression of the CXCR4 gene was significantly lower ( $p < 0.001$ , Figure 3). However, compared with the H/R group, the expression of the miRNA-155 gene in the miRNA-inhibitor group was significantly lower while the expression of the CXCR4 gene was significantly higher ( $p < 0.001$ , Figure 3).

### Effects of miRNA-155 on invasion and migration of cells

The transwell cell invasion and wound healing experiments showed that compared with the NC group, the number of transmembrane cells and the rate of wound healing were significantly lower in the H/R, BL, miRNA-155 mimics, miRNA-NC and inhibitor NC groups ( $p < 0.001$ , Figure 4), and that compared with the H/R group, the number of

transmembrane cells and the rate of wound healing were significantly higher in the miRNA-inhibitor group ( $p < 0.001$ , Figure 4).

### Effects of miRNA-155 on related proteins and entry of p-AKT into the nucleus

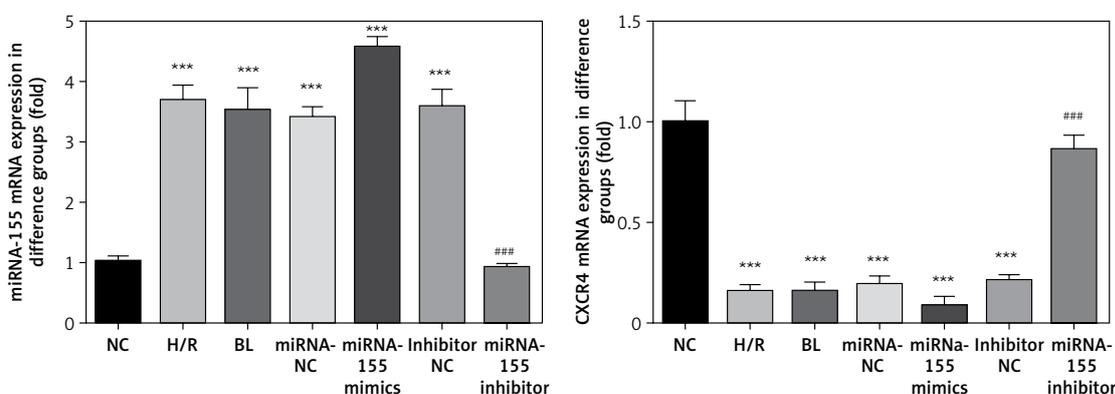
Compared with the NC group, the expression of the CXCR4 and p-AKT proteins and the amount of p-AKT protein entering the nucleus in the H/R, BL, miRNA-155 mimics, miRNA-NC and inhibitor NC groups were significantly lower ( $p < 0.001$ , Figure 5). However, compared with the H/R group, the expression of the CXCR4 and p-AKT proteins and the amount of p-AKT protein entering the nucleus were significantly higher in the miRNA-inhibitor group ( $p < 0.001$ , Figure 5).

### Effects of Sal B on expression levels of miRNA-155 and CXCR4 genes in cells in various groups

Compared with the NC group, the expression of the miRNA-155 gene was significantly higher in the H/R group and Sal B + miRNA-155 group, while the expression of the CXCR4 gene was significantly lower ( $p < 0.001$ , Figure 6). However, compared with the H/R group, the expression of the miRNA-155 gene was significantly lower in the Sal group, while the expression of the CXCR4 gene was significantly higher ( $p < 0.001$ , Figure 6).

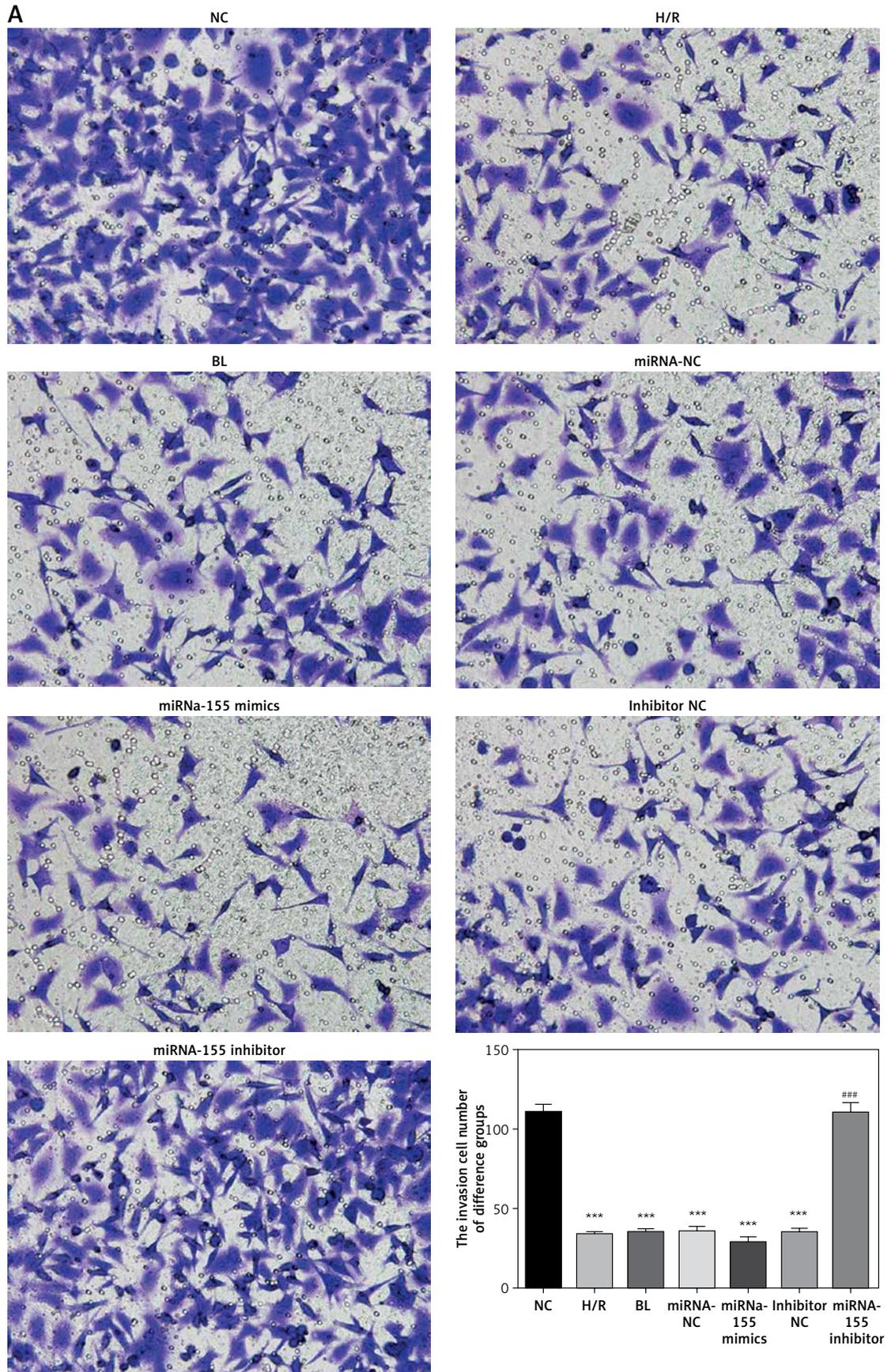
### Effects of Sal B on invasion and migration of cells

The transwell cell invasion and wound healing experiments showed that compared with the NC group, the number of transmembrane cells and the rate of wound healing were significantly lower in the H/R group and the Sal B + miRNA-155 group ( $p < 0.001$ , Figure 7); compared with the H/R group, the number of transmembrane cells and the rate of wound healing in the Sal B group were markedly higher ( $p < 0.001$ , Figure 7).



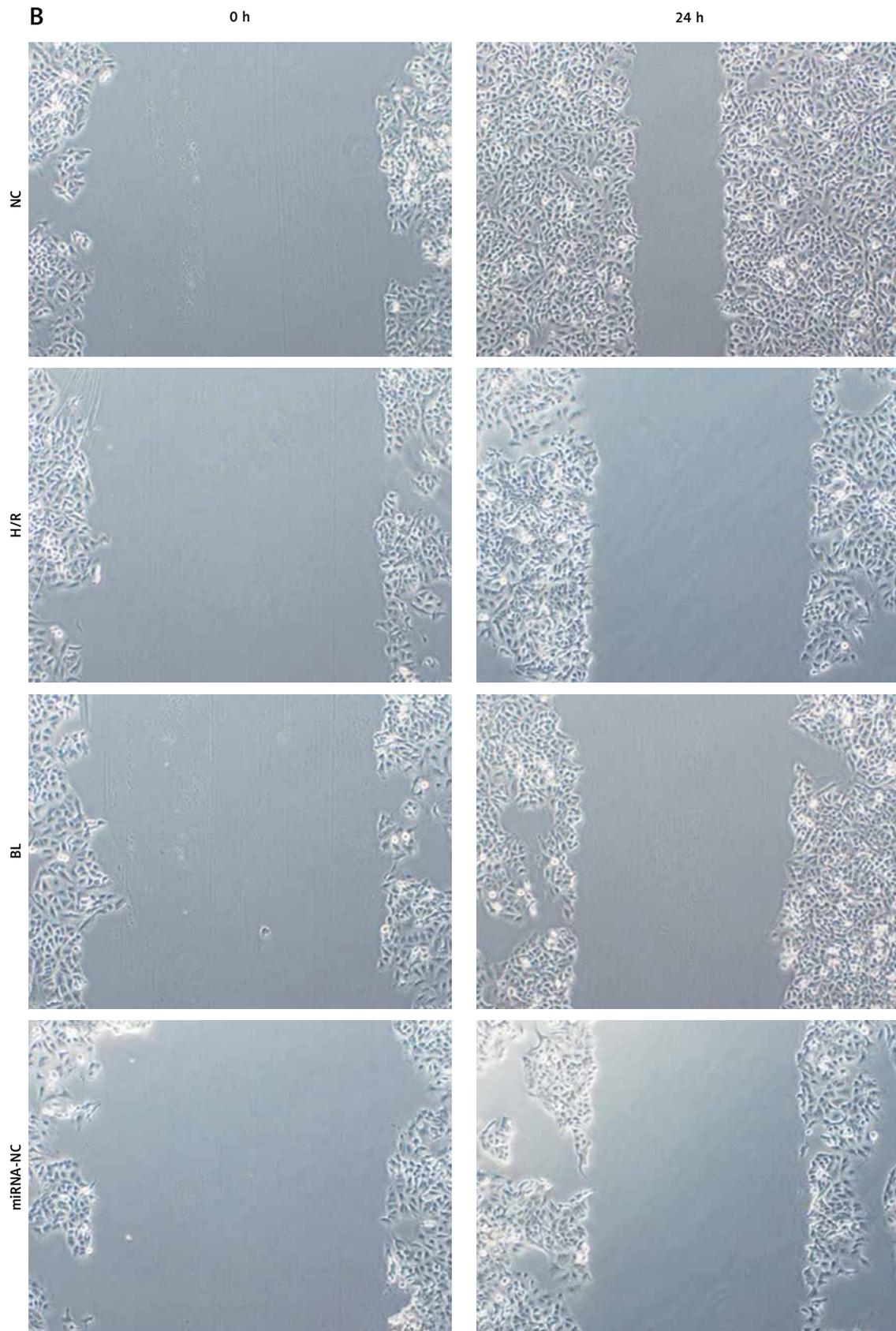
**Figure 3.** miRNA-155 and CXCR4 gene expression in difference cell groups by RT-PCR

\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.

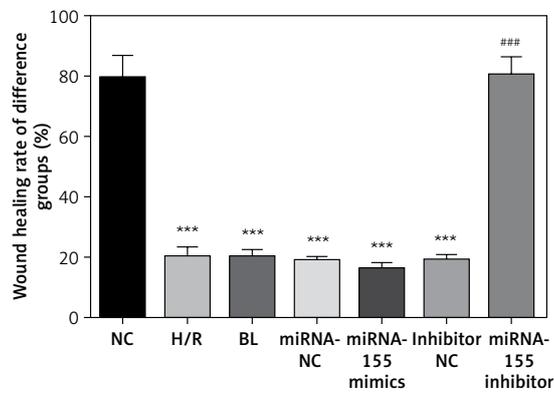
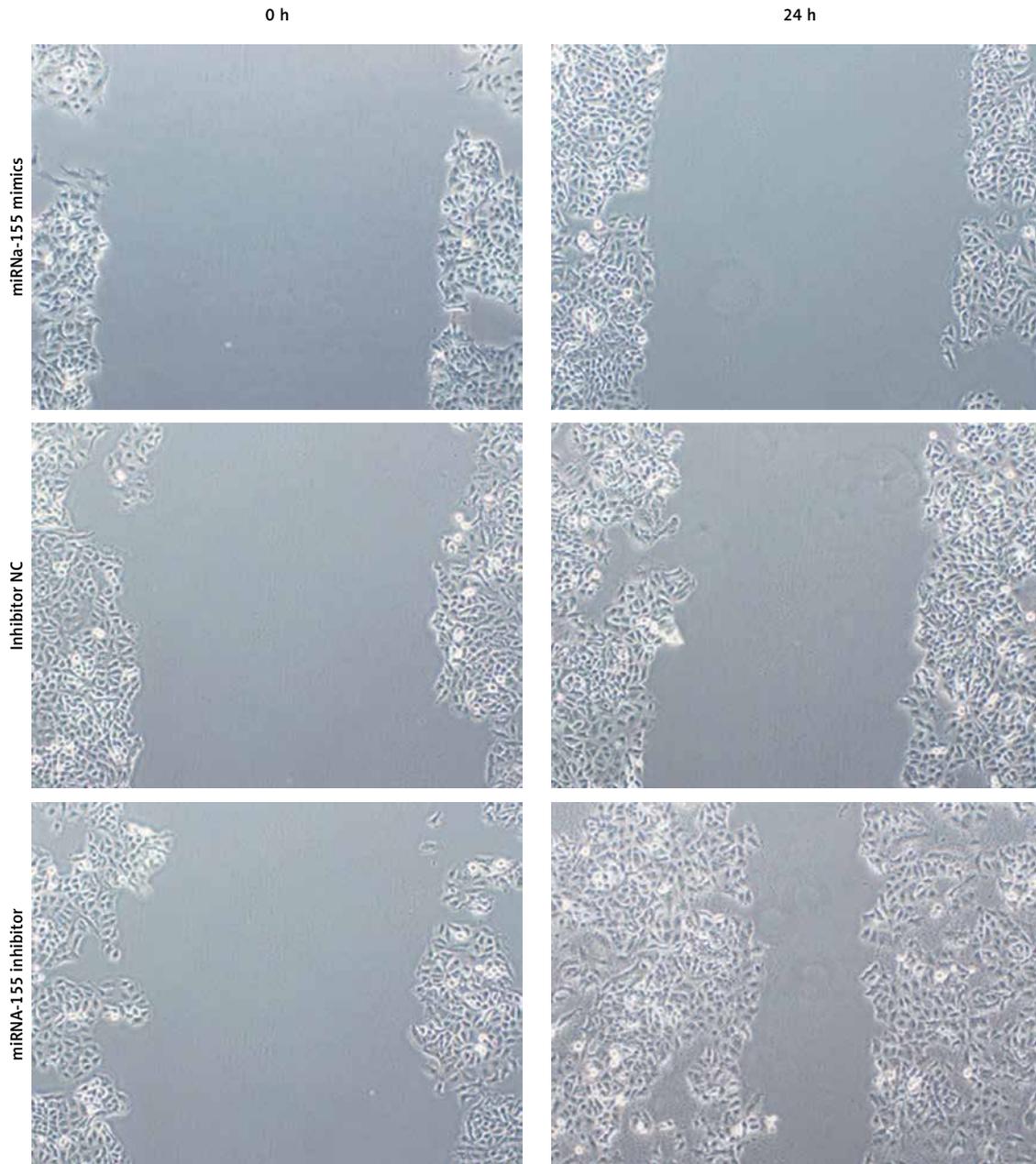


**Figure 4.** miRNA-155 effect on cell invasion and migration. **A** – miRNA-155 effect on cell invasion by transwell assay

\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.

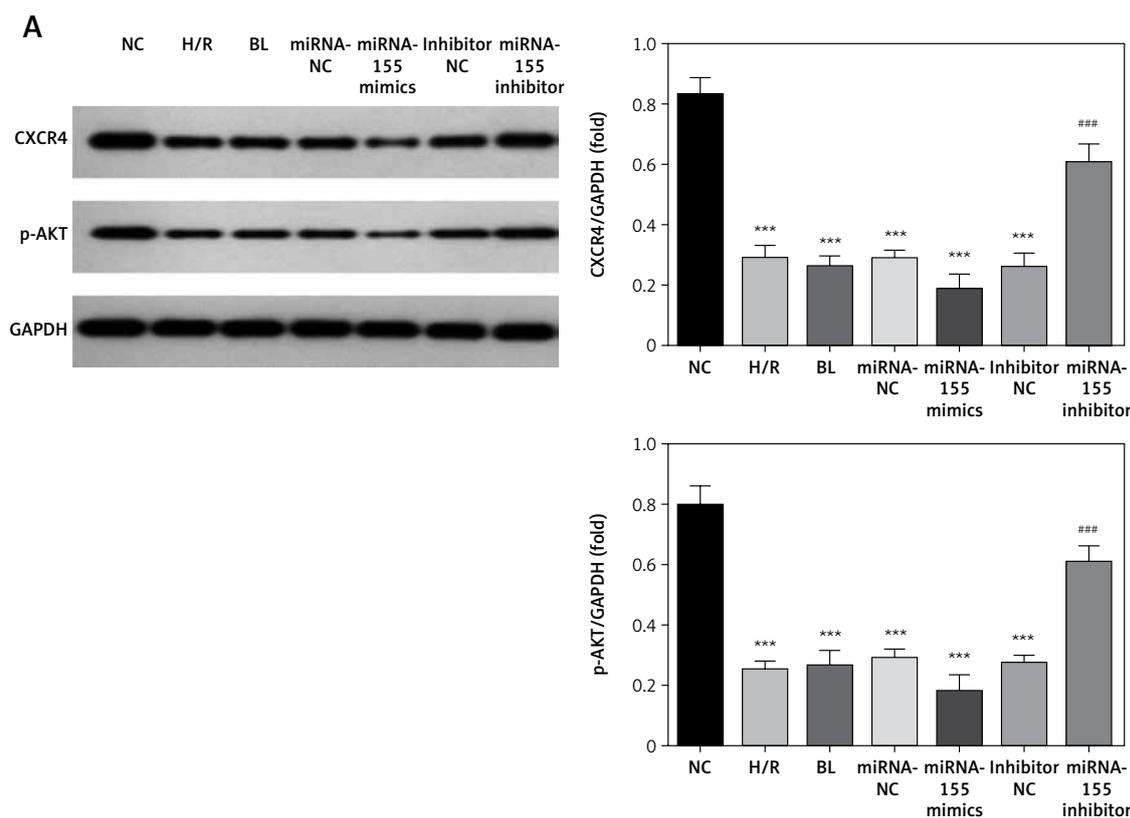


**Figure 4.** Cont. **B** – miRNA-155 effect on cell migration by wound healing assay  
\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.



**Figure 4. Cont. B** – miRNA-155 effect on cell migration by wound healing assay

\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.



**Figure 5.** CXCR4 and p-AKT protein expression by WB assay and p-AKT nucleation volume. **A** – CXCR4 and p-AKT proteins expression by WB assay

\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.

### Effects of Sal B on related proteins and entry of p-AKT into the nucleus

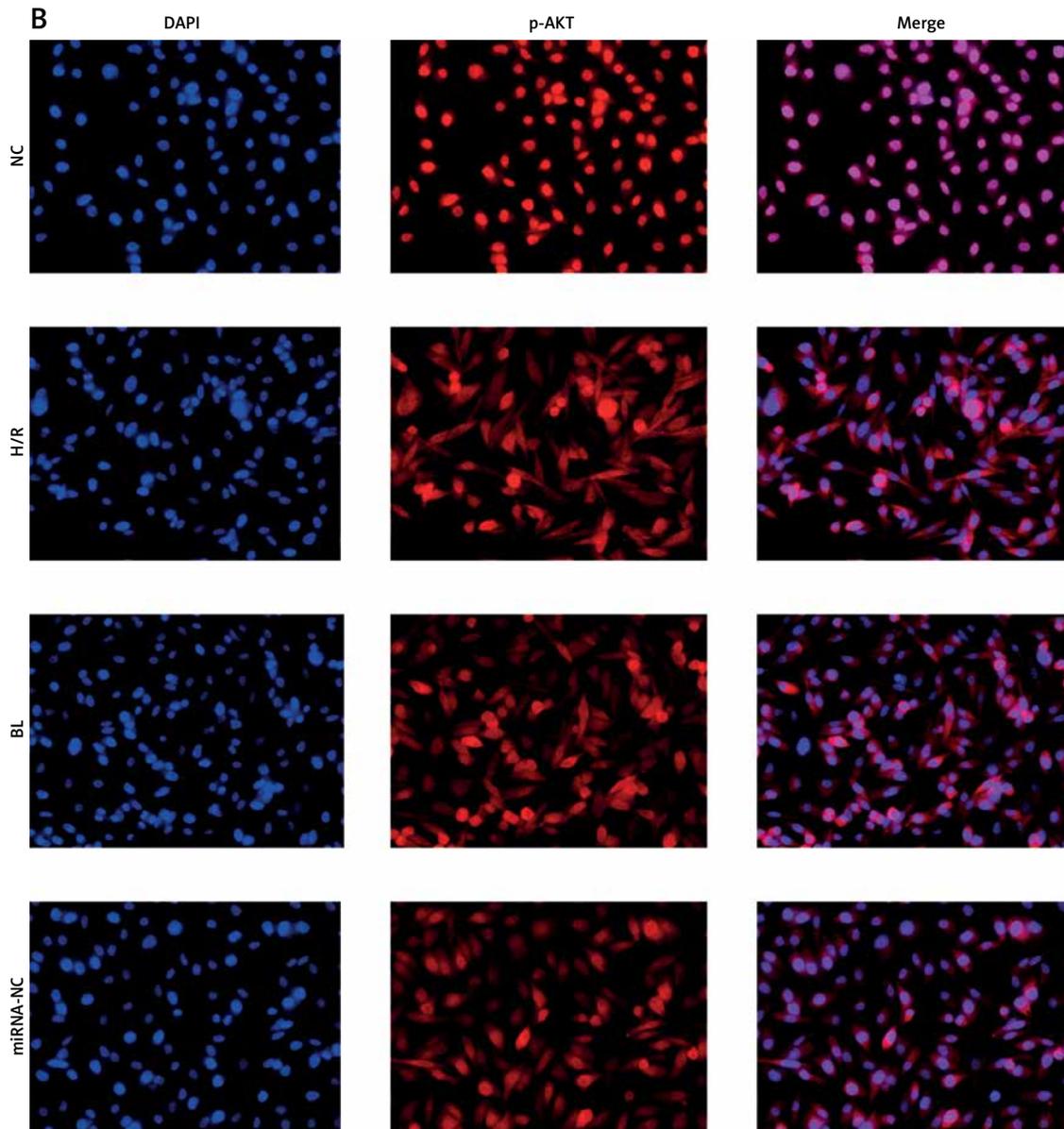
Compared with the NC group, the expression of CXCR4 and p-AKT proteins and the amount of p-AKT protein entering the nucleus in the H/R group and the Sal B + miRNA-155 group were significantly lower ( $p < 0.001$ , Figure 8); however, compared with the H/R group, the expression of CXCR4 and p-AKT proteins and the amount of p-AKT protein entering the nucleus in the Sal group were significantly higher ( $p < 0.001$ , Figure 8).

### Discussion

PE represents an idiopathic hypertensive syndrome presenting with hypertension and proteinuria, etc. after 20 weeks of gestation in women with normal blood pressure. It can lead to serious maternal complications and is one of the main causes of maternal and perinatal death [16]. The incidence of PE in Europe and the United States at present is low (approximately 2–5%), while it is more common in developing countries, with an incidence of about 10%. PE accounts for 9% of maternal mortality in Asia and Africa [17]. Among 10 million pregnant women worldwide every year, gestational hypertension, especially PE,

leads to the death of 76,000 pregnant women and 500,000 fetuses or neonates [18]. The American College of Cardiology has also included PE among the risk factors for cardiovascular disease, and recommended that patients with a history of PE be understood and their lifestyles be improved [19, 20]. However, the mechanisms of pathogenesis underlying PE remain unclear, and there is still a lack of effective treatment options in clinical practice. Pregnancy has to be terminated early in many cases, causing pregnancy failure, and many epidemiological studies have also confirmed that the development of PE may have long-term adverse consequences in the offspring [21, 22]. The mechanisms of pathogenesis underlying PE may involve many factors, such as placental ischemia and hypoxia, inflammatory responses, changes in the PE-associated signaling pathways, trophoblastic infiltration abnormalities, abnormal maternal-fetal interface immunity, local coagulation of placenta and imbalanced anticoagulant mechanisms, and the abnormal expression profiles of miRNAs [23, 24].

Since the discovery of the first miRNA in *Caenorhabditis elegans* by Lee *et al.* in 1993 [25], more than 2,000 miRNAs have been discovered, and bioinformatics has predicted that miRNA may



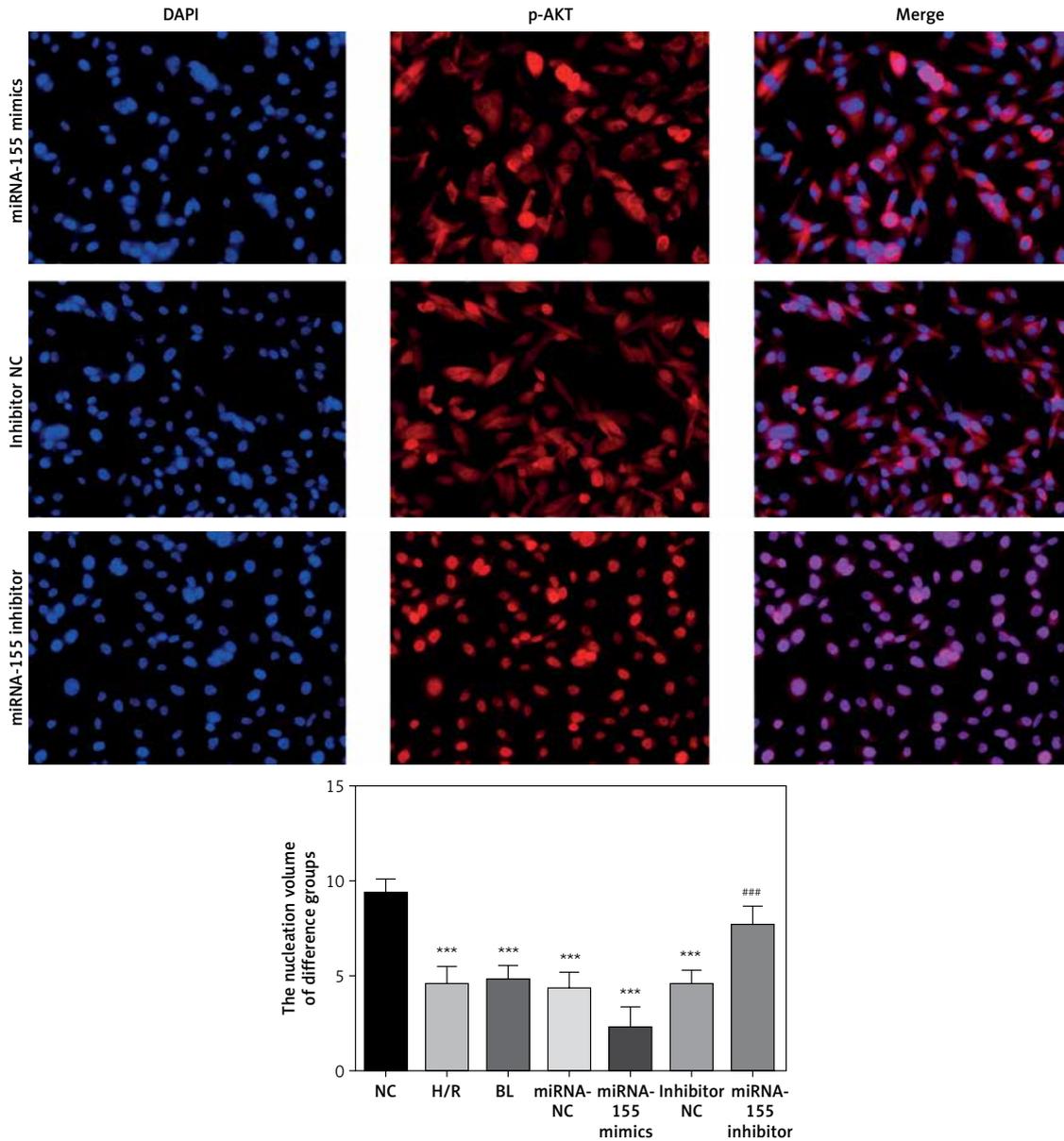
**Figure 5. Cont. B – p-AKT nucleation volume of different groups by cell immunofluorescence**

\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.

regulate 60% of genes encoding proteins within the human genome [26]. Great strides have been made in research on miRNAs in inflammation and tumors, while research on eclampsia-associated miRNAs has just started. miRNAs can regulate the post-translational expression of genes by undergoing complementary pairing with the 3' non-coding region (3'UTR) of mRNA completely or incompletely, and plays an important role in the differentiation, development, proliferation, invasion, apoptosis and other biological processes of cells [27]. Encoded by the BIC gene, the miRNA-155 gene is located on human chromosome 21q21. 3, and is a miRNA closely associated with inflammation, tumors and immune regulation [28]. Recent studies have shown that miRNA-155 can target

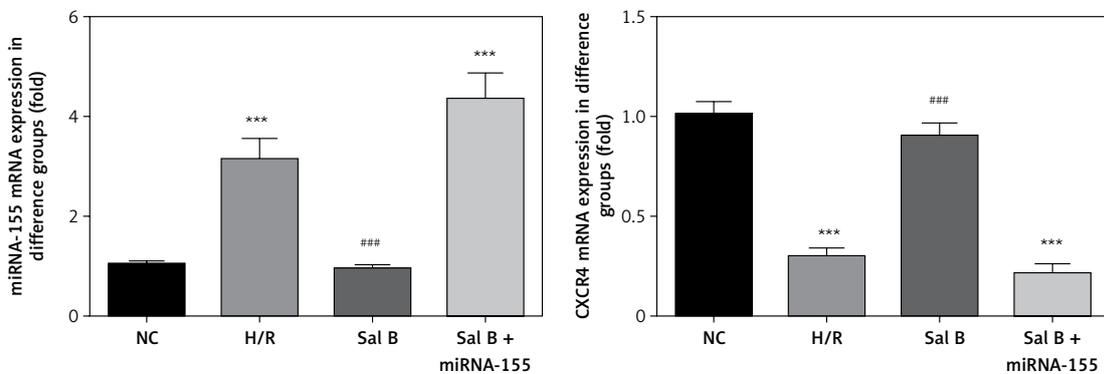
the regulation of expression of CXCR4, affect the proliferation and angiogenesis of cancer cells and increase the invasiveness of tumor cells in such diseases as gastric cancer and breast cancer [29, 30]. CXCR4, which is also known as chemokine receptor 4, is located on the human chromosome 2q21, and consists of 352 amino acids. It can be expressed on the membranes of various cells, including trophoblast cells, and is expressed in the villus tissues throughout pregnancy [31].

Sal B is an active ingredient from the traditional Chinese medicine *Salvia miltiorrhiza*. Pharmacological studies have shown that Sal B can regulate the vascular tone by blocking the  $\beta$ -receptors, reducing the calcium influx, antagonizing endothelin, and increasing the generation of NO in the



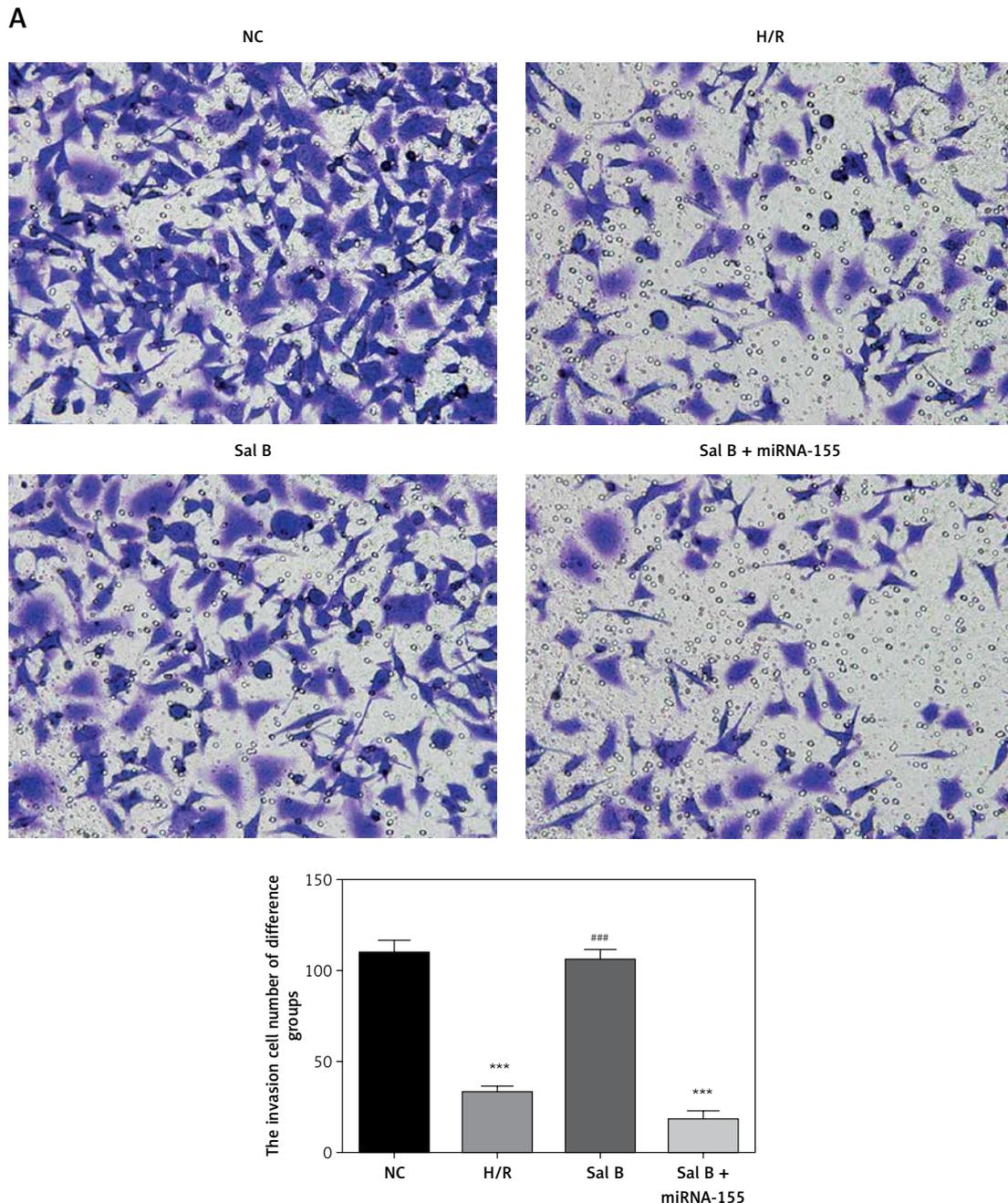
**Figure 5. Cont. B** – p-AKT nucleation volume of different groups by cell immunofluorescence

\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.



**Figure 6.** miRNA-155 and CXCR4 mRNA expression in different groups by RT-PCR assay

\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.



**Figure 7.** Cell invasion and migration in different groups by transwell and migration. **A** – Cell invasion of different groups by transwell assay

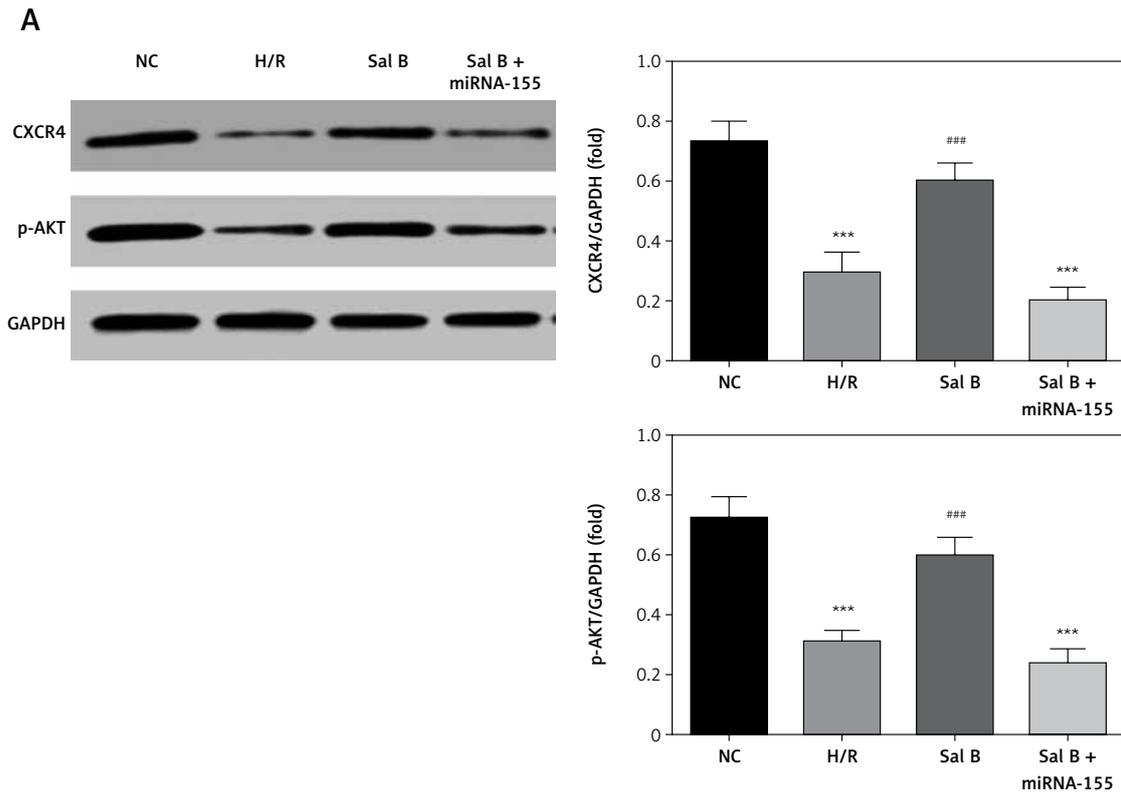
\*\*\* $P < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.

platelets, etc., thus increasing the blood flow [32, 33]. These show that Sal B exhibits good effects in combating platelet aggregation, dilating small arteries, and improving microcirculation and hemorheology, and that it can improve the invasion and migration of chorionic trophoblast cells.

This study demonstrated that Sal B exhibited marked effects on improving the blood pressure and urine protein and promoting the development and maturation of young rats, suggestive of the good effects of Sal B in improving the vascular

tension of the placenta and improving hypoxia and ischemia of cells. In the meantime, it was found that Sal B could lead to an increased concentration of NO in the plasma and enhanced invasion and migration of trophoblast cells. The cell experiments showed that Sal B could restore the injury of invasion and migration of the chorionic trophoblast cells as a result of simulation of the hypoxia model of PE. The mechanisms underlying this may be associated with promoted CXCR4 expression levels and p-AKT protein activity and an





**Figure 8.** CXCR4 and p-AKT protein expression by WB assay and p-AKT nucleation volume. **A** – CXCR4 and p-AKT protein expression by WB assay

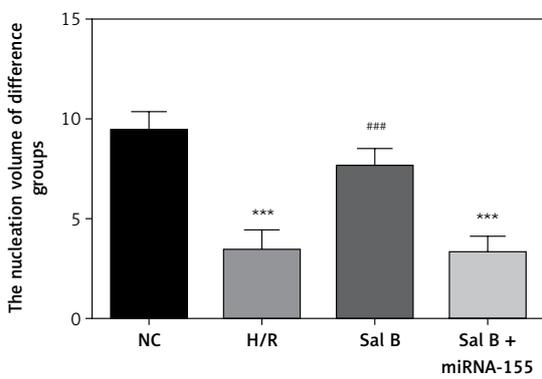
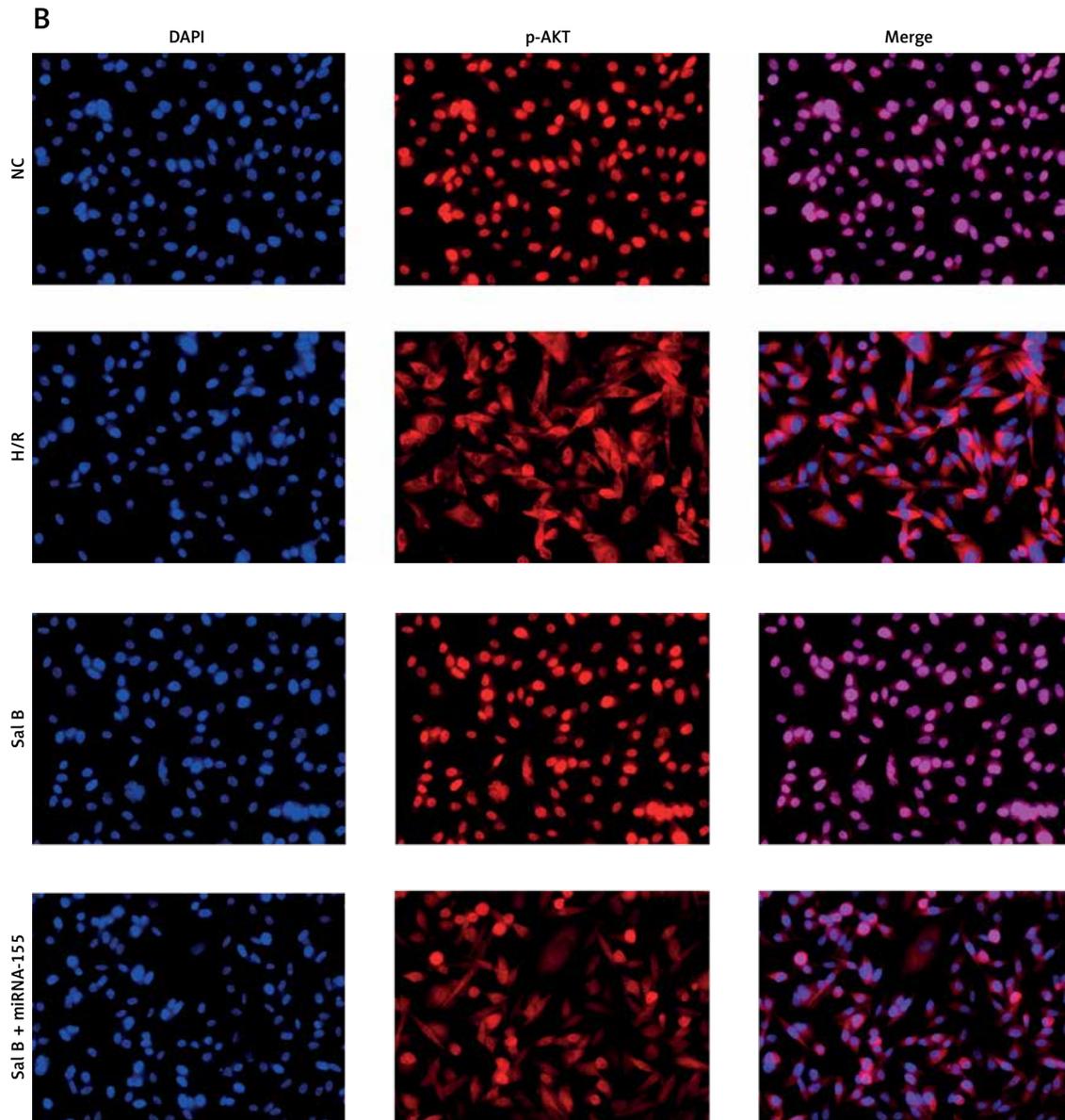
\*\*\* $p < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.

increased nuclear load through inhibition of miRNA-155.

In conclusion, this study, through *in vivo* and *in vitro* experiments, demonstrated that Sal B could improve the pathological changes in PE by inhibiting miRNA-155, and its mechanisms may include activation of the CXCR4 /PI3K/AKT signaling pathway to affect such biological functions of trophoblasts as invasion and migration.

### Conflict of interest

The authors declare no conflict of interest.



**Figure 8.** Cont. **B** – p-AKT nucleation volume of different groups by cell immunofluorescence  
\*\*\* $p < 0.001$ , compared with NC group; ### $p < 0.001$ , compared with H/R group.

## References

- Lain KY, Roberts JM. Contemporary concepts of the pathogenesis and management of preeclampsia. *J Am Med Assoc* 2002; 287: 3183-6.
- Saito S, Shiozaki A, Nakashima A, Sakai M, Sasaki Y. The role of the immune system in preeclampsia. *Mol Aspects Med* 2007; 28: 192-209.
- Murphy MS, Casselman RC, Tayade C, Smith GN. Differential expression of plasma microRNA in preeclamptic patients at delivery and 1 year postpartum. *Am J Obstet Gynecol* 2015; 213: 367e1-9.
- Lazar L, Rigo J. The role of microRNA in pathogenesis of preeclampsia-miRNA network analysis. *Pregnancy Hypertens* 2013; 3: 99.
- Li XL, Li CF, Dong X, Gou W. MicroRNA-155 inhibits migration of trophoblast cells and contributes to the pathogenesis of severe preeclampsia by regulating endothelial nitric oxide synthase. *Mol Med Reports* 2014; 10: 550-4.
- Dai Y, Qiu Z, Diao Z, et al. MicroRNA-155 inhibits proliferation and migration of human extravillous trophoblast derived HTR-8/SVneo cells via down-regulating cyclin D1. *Placenta* 2012; 33: 824-9.
- Wang L, Li X, Zhao Y, et al. Insights into the mechanism of CXCL12-mediated signaling in trophoblast functions and placental angiogenesis. *Acta Biochim Biophys Sin (Shanghai)* 2015; 47: 663-72.
- Warner JA, Zvezdaryk KJ, Day B, Sullivan DE, Pridjian G, Morris CA. Human cytomegalovirus infection inhibits CXCL12-mediated migration and invasion of human extravillous cytotrophoblasts. *Virology* 2012; 9: 255.
- Ho JH, Hong CY. Salvianolic acids: small compounds with multiple mechanisms for cardiovascular protection. *J Biomed Sci* 2011; 18: 30.
- Yu LJ, Zhang KJ, Zhu JZ, et al. Salvianolic acid exerts cardioprotection through promoting angiogenesis in animal models of acute myocardial infarction: preclinical evidence. *Oxid Med Cell Longev* 2017; 2017: 8192383.
- Liu M, Xu H, Zhang L, et al. Salvianolic acid B inhibits myofibroblast transdifferentiation in experimental pulmonary fibrosis via the up-regulation of Nrf2. *Biochem Biophys Res Commun* 2018; 495: 325-31.
- Sha W, Zhou Y, Ling ZQ, et al. Antitumor properties of Salvianolic acid B against triple-negative and hormone receptor-positive breast cancer cells via ceramide-mediated apoptosis. *Oncotarget* 2018; 9: 36331-43.
- Li Y, Wang L, Dong Z, et al. Cardioprotection of salvianolic acid B and ginsenoside Rg1 combination on subacute myocardial infarction and the underlying mechanism. *Phytomedicine* 2019; 57: 255-61.
- Yu J, Chen R, Tan Y, et al. Salvianolic acid B alleviates heart failure by inactivating ERK1/2/GATA4 signaling pathway after pressure overload in mice. *PLoS One* 2016; 11: e0166560.
- Leach RE, Kilburn BA, Petkova A, Romero R, Armant DR. Diminished survival of human cytotrophoblast cells exposed to hypoxia/reoxygenation injury and associated reduction of heparin-binding EGF-like growth factor. *Am J Obstet Gynecol* 2008; 198: 471.e1-7.
- Ghulmiyyah L, Sibai B. Maternal mortality from preeclampsia / eclampsia. *Semin Perinatol* 2012; 36: 56-9.
- Duley L. The global impact of pre-eclampsia and eclampsia. *Semin Perinatol* 2009; 33: 130-7.
- Khowaja AR, Mitton C, Bryan S, Magee LA, Bhutta ZA, von Dadelszen P. Economic evaluation of community level interventions for pre-eclampsia (CLIP) in south Asian and African countries: a study protocol. *Implement Sci* 2015; 10: 76.
- Mosca L, Benjamin EJ, Berra K, et al.; American Heart Association. Effectiveness-based guidelines for the prevention of cardiovascular disease in women-2011 update: a guideline from the American Heart Association. *J Am Coll Cardiol* 2011; 57: 1404-23.
- Seely EW, Rich-Edwards J, Lui J, et al. Risk of future cardiovascular disease in women with prior preeclampsia: a focus group study. *BMC Pregnancy Childbirth* 2013; 13: 240.
- Davis EF, Lazdam M, Lewandowski AJ, et al. Cardiovascular risk factors in children and young adults born to preeclamptic pregnancies: a systematic review. *Pediatrics* 2012; 129: E1552-61.
- Lawlor DA, Macdonald-Wallis C, Fraser A, et al. Cardiovascular biomarkers and vascular function during childhood in the offspring of mothers with hypertensive disorders of pregnancy: findings from the Avon Longitudinal Study of Parents and Children. *Eur Heart J* 2012; 33: 335-45.
- Chaiworapongsa T, Chaemsaitong P, Yeo L, Romero R. Pre-eclampsia part 1: current understanding of its pathophysiology. *Nat Rev Nephrol* 2014; 10: 466-80.
- Chen DB, Wang W. Human placental microRNAs and preeclampsia. *Biol Reprod* 2013; 88: 130.
- Lee RC, Feinbaum RL, Ambros V. The *c. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 1993; 75: 843-54.
- Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009; 19: 92-105.
- Bushati N, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol* 2007; 23: 175-205.
- Garo LP, Murugaiyan G. Contribution of MicroRNAs to autoimmune diseases. *Cell Mol Life Sci* 2016; 73: 2041-51.
- Ma Z, Ma Y, Xia Q, et al. MicroRNA-155 expression inversely correlates with pathologic stage of gastric cancer and it inhibits gastric cancer cell growth by targeting cyclin D1. *J Cancer Res Clin Oncol* 2016; 142: 1201-12.
- Liang Z, Wu H, Reddy S, et al. Blockade of invasion and metastasis of breast cancer cells via targeting CXCR4 with an artificial microRNA. *Biochem Biophys Res Commun* 2007; 363: 542-6.
- Zhao HB, Tang CL, Hou YL, et al. CXCL12 /CXCR4 axis triggers the activation of EGF receptor and ERK signaling pathway in CsA-induced proliferation of human trophoblast cells. *PLoS One* 2012; 7: e38375.
- Yang K, Luo Y, Lu S, et al. Salvianolic acid B and ginsenoside re synergistically protect against Ox-LDL-induced endothelial apoptosis through the antioxidative and antiinflammatory mechanisms. *Front Pharmacol* 2018; 9: 662.
- Liu CS, Chen NH, Zhang JT. Protection of PC12 cells from hydrogen peroxide-induced cytotoxicity by salvianolic acid B, a new compound isolated from *Radix Salviae miltiorrhizae*. *Phytomedicine* 2007; 14: 492-7.