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# The involvement of miR-155 in blood pressure regulation in pregnant hypertension rat via targeting FOXO3a

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**Abstract. – OBJECTIVE:** Pathogenesis factor of pregnant hypertension is still unclear and lacks of effective treatment. MiR-155 is a recently discovered miRNA molecule with differential expression in pregnant hypertension, which participates in the disease regulation. As a downstream target gene of miR-155, FOXO3a is correlated with blood pressure regulation. We investigated the regulatory role and mechanism of miR-155 in pregnant hypertension.

**MATERIALS AND METHODS:** We established a pregnant hypertension rat model, on which miR-155 inhibitor or FOXO3a siRNA was applied, followed by HE staining, 24 h urea protein, blood pressure and serum creatine assay to evaluate disease severity.

**RESULTS:** MiR-155 expression was significantly elevated in model rats, accompanied by a reduction of the FOXO3a level. MiR-155 inhibitor suppressed miR-155 expression, increased FOXO3a level and placental tissue morphology by HE staining, and depressed blood pressure as well as serum creatine level. Downregulation of FOXO3a by specific siRNA resulted in opposite effects. These results illustrated the miR-155 mediated FOXO3a expression in pregnant hypertension.

**CONCLUSIONS:** The inhibition of miR-155 improves the damage of pregnant hypertension via the upregulation of FOXO3a, which provides academic leads for the future therapy of pregnant hypertension.

*Key Words:*
MiR-155, FOXO3a, Pregnant hypertension.

## Introduction

Pregnant hypertension is associated with pathogenic factors. Previous study showed the close relationship between placenta and pregnant hypertension. Abnormal differentiation and apoptosis of placental trophocytes are basis for series of clinical symptoms in pregnant hypertension. When placenta is discharged, the hypertension rapidly disappeared[1,2]. So far little knowledge on its regulatory mechanism has restricted the treatment of pregnant hypertension. Moreover, the safety requires to be fully considered in the meantime with treatment. Currently, no effective treatment is reported for pregnant hypertension. The identification of potential treatment target for pregnant hypertension is, thus, of critical importance. Recent investigations[3] showed significant roles of microRNA (miR) in onset and progression of multiple diseases. MiR belongs to the group of noncoding small molecule single stranded RNA with 18-24bp length. It can bind with 3'-untranslated region of target mRNA via complete or incomplete binding, and result in selective degradation under the direction of RNA exonuclease, thus inhibiting or activating downstream genes[4]. MiR thus contributes to vital function in basic cellular biological processes including cell proliferation, apoptosis, differentiation and migration[5]. Previous evidence showed over-expression of certain miR molecules in mouse and human placenta, indicating the potential role of miRs in placenta development and their abnormal expression in placental function and pregnancy outcomes[6]. Early study revealed the involvement of miR-155 in blood cell genesis, tumor and inflammatory vascular diseases[7]. In a comparative study recruiting 59 PE patients and 40 healthy pregnant placenta, miR-155-5p expression was significantly elevated in patients[8]. Forkhead box O3a (FOXO3a) represents an important member of FOXO subfamily, and has been confirmed to be a target gene of miR-155 in previous studies[9]. FOXO subfamily is widely expressed in various adult tissues or organs, including skeletal muscle, nervous system. Early finding showed that the activation of

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FOXO3a-PGC-1α signal pathway improved high fat diet induced hypertension[10]. FOXO3a is critical for maintaining cardiac function, regulating blood pressure and anti-oxidative stress, but its expression or functional role in pregnant hypertension remains to be elucidated. Therefore, we aimed to investigate the expression level of miR-155 and FOXO3a in pregnant hypertension and related mechanisms.

## Materials and Methods

### Materials Reagent

Chloralose and urethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Total protein extraction kit was collected from Kaiji Biotech (Shanghai, China). Reverse transcription and Real-time quantitative PCR kits were purchased from Toyobo (Osaka, Japan). HE staining kit was bought from Zhongshan Jinqiao (Shanghai, China). Beta-actin internal reference antibody was acquired from Kangcheng Bio (Shanghai, China). FOXO3a antibody was purchased from Abcam (Cambridge, MA, USA). Rabbit anti-mouse IgG (H+L) and rabbit anti-mouse IgG (H+L) were bought from Proteintech (Rosemont, IL, USA). SYBR Green Polymerase Chain Reaction (PCR) Master Mix was obtained from Toyobo (Osaka, Japan). MiR-155 inhibitor and negative control sequence were synthesized from Gimma (Shanghai, China). Electrochemiluminescence (ECL) chromogenic substrate was purchased from Beyotime (Beijing, China).

### Major Equipment

Metabolic cage was provided by Boxun (Changchun, Jilin, China). Gel imaging system UVP Multispectral Imaging System (Hercules, CA, USA), PS-9 semi-dry transfer electrophoresis was purchased from Jingmai (Shanghai, China). Thermo-354 microplate reader was from Thermo Fisher (Waltham, MA, USA). Non-invasive blood pressure meter was purchased from Yuyan Instrument (Shanghai, China). HE staining and slices were prepared by Jinan Maternity and Childcare Hospital (Shandong China). Coulter DXC800 automatic biochemical analyzer was purchased from Beckman (Brea, CA, USA).

### Experimental Animals and Grouping

2-month age male and female SD rats (N=30 each) were provided by Laboratory Animal Center of Shangdong University. Animals were assigned into normal pregnant group, nitric oxide synthase inhibitor L-NAME treated pregnant hypertension group, L-NAME + miR-155 inhibitor treated group, miR control group, FOXO3a knockdown lentivirus transfection group and lentiviral control group.

### Model Preparation

A total of 40 healthy SD female rats were mated with males at 1:1 ratio. Vaginal smears were collected and checked under the microscope. The day on which active sperm was identified was denoted as day 0. On day 12, basal blood pressures of all pregnant rats were measured, and 24 h urine volume was measured by a metabolic cage. On day 14, pregnant rats were randomly divided into four groups: normal pregnant group, L-NAME treated pregnant hypertension group, L-NAME + scramble, and L-NAME+miR-155 inhibitor group. On pregnant day 14, control group received 80 mg/kg/d L-NAME via gavage to generate rat pregnant hypertensive model. Normal pregnant group received equal volume of saline via gavage. On the second day of L-NAME gavage (gestation day 16), blood pressure of all pregnant rats was measured. Those animals that fitted diagnostic criteria were recruited and received 80 mg/kg/d L-NAME for 7 days. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Jinan Maternity and Childcare Hospital (Shandong China).

### Drug Delivery

After confirming successful generation of pregnant hypertension model, scramble or miR-155 inhibitor (5 mg/kg) was delivered by tail vein injection for 4 consecutive days.

### HE Staining for Placenta Tissues

Placental tissues were fixed in 4% paraformaldehyde for 24 h, and were dehydrated in gradient ethanol, followed by xylene treatment and embedding. Those placental tissues were sectioned into paraffin-based slices with 4 μm thickness. Slices were de-waxed, rehydrated, stained in hematoxylin, and rinsed in tap water. After differentiation in 1% HCl-ethanol and rinsed under tap water, eosin staining was performed. Slices were rinsed in tap water and dehydrated in gradient ethanol. After processed in xylene, slices were mounted with coverslips by neutral resin.

### Total RNA Extraction

100 mg placental tissues were mixed with 1 mL TRIzol for 5 min iced incubation. The tissue lysate

was mixed by pipetting, and was removed into 1.5 mL tubes. Each tube was added with 0.2 mL chloroform for 15 s shaking, followed by 3 min room temperature incubation. The mixture was centrifuged at 12000 g under 4°C for 10 min. The upper aqueous phase was carefully removed into new tubes, which were added with 0.5 mL isopropanol for 10 min room temperature incubation. The mixture was then centrifuged at 12000 g under 4°C for 10 min. The upper phase was discarded, and 1 mL ethanol was added for three times of rinsing. The supernatant was carefully removed, and mRNA was re-suspended into 20 μL diethyl pyrocarbonate (DEPC) water.

### Real-Time Quantitative PCR

MiR-155 primers were synthesized by Sigma-Aldrich using the following sequences[11]: Forward, 5'-TTGAA TTCTA ACACC TTCGT GGCTA CAGAG-3'; Reverse, 5'-TTAGA TCTCA TTTAT CGAGG GAAGG ATTG-3'. U6 was used as the internal reference gene: forward, 5'-CTCGC TTCGG CAGCA CA-3'; reverse, 5'-AACGC TTCAC GAATT TGCGT-3'. Reverse transcription was performed in a 20 μL system following the manual instruction of test kit. PCR was performed in a 50 μL system following manual instruction. Reaction conditions were: 50°C for 30 min, and 95°C 5 min, followed by 40 cycles each consisting of 95°C 30 s, 55°C 30 s and 72°C 50 s, and ended with 72°C elongation for 5 min. After reaction ended, Real-time PCR amplification curve and melting curve were determined, and relative expression was calculated by comparing Ct values of target gene and internal reference gene. Gene expression was quantified by $2^{-\Delta\Delta Ct}$ approach.

### Blood Pressure Measurement

Noninvasive blood pressure meter was used to quantify tail artery pressure of pregnant rats at gestation day 12, day 16 and day 20. In brief, pregnant rats were fixed in the apparatus, which was adjusted based on the size of pregnant rat to keep it comfortable. The air bag was installed in the middle part of rat-tail for connecting pressure meter and pulsing sensor. When animals were quiet and stable pulsing wave was shown on the monitor, rat-tail artery pressure was measured. A second measurement was taken after stabilization of pulsing waveform after one measurement. Each rat was measured for 5 times and the average value was calculated. Those animals with systolic pressure increased by more than 20 mmHg, and absolute value higher than 115 mmHg were confirmed as successful generation of hypertensive model.

### Serum Creatinine Level

Full blood samples were centrifuged to obtain the serum, and the creatinine level was measured by automatic biochemical analyzer. Serum creatinine (Scr) level was determined by alkaline picric acid colorimetry, following the manual instruction of test apparatus and test kit.

### 24 h Urine Protein Assay

All pregnant rats were fed with food and water *ad libitum*. On gestation day 12, day 16 and day 20, a metabolic cage was used to collect 24 h urine volume from all groups. 5 mL samples were collected from each animal for measuring 24 h urine protein on an automatic biochemical analyzer.

### Western Blot

Placental tissues were homogenized to extract total protein. Proteins were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred into polyvinylidene difluoride (PVDF) membranes, blocked with 5% nonfat milk for 1 h, washed with phosphate-buffered solution and Tween 20 (PBST) for 3 times, and incubated with primary antibody (1:1000) overnight at 4°C. The membrane was then washed with PBST for 30 min, followed with incubation with secondary antibody (1:5000) for 60 min. After the membrane was washed three times with PBST, chemiluminescence detection reagent was used to develop and fix.

### Statistical Analysis

All data were obtained from at least three times of independent experiments. Data were presented as mean±standard deviation (SD). Two-sample comparison was performed by Student *t*-test. One-way analysis of variance (ANOVA) with Tukey's post-hoc test was performed for comparing among multiple samples, followed by SNK approach in paired comparison between groups. A statistical significance was defined when $p<0.05$.

## Results

### Pregnant, Grouping and General Condition of Rats

Pregnant hypertensive rats model treated by L-NAME was measured in comparison of blood

pressure before and after the treatment. We found that the blood pressure was significantly elevated, and rats were diagnosed as hypertension. All individuals were thus recruited into the experiment. During experimental period, all rats showed satisfactory growth status without death.

### MiR-55 Expression in Pregnant Hypertension

In this study, we utilized PCR to quantify miR-155 expression level in rat placenta tissues. As shown in Figure 1, miR-155 level in L-NAME treated rats was significantly elevated ($p<0.05$). However, miR-155 inhibitor remarkably decreased miR-155 level in L-NAME treated rats.

### FOXO3a Expression Assay

With reference to database including Target Scan and miRna, we found that FOXO3a was target gene of miR-155. Evidence showed that over-expression of miR-155 had potent effect of cancer induction. Using Western blot, we measured expression level of downstream protein FOXO3a. As shown in Figure 2, FOXO3a level presented an opposite trend with miR-155. Comparing to control group, FOXO3a expression in L-NAME treated rats was significantly depressed ($p<0.05$). FOXO3a siRNA further suppressed FOXO3a expression.

### MiR-155 Negatively Regulated FOXO3a Expression

The relation between FOXO3a and direct target gene of miR-155 was analyzed and the result showed the complementary binding at 3'-UTR of FOXO3a (Figure 3A). After knocking down miR-155 level, FOXO3a expression level was significantly up-regulated compared with control group. These results showed that miR-155 participated in the regulation of FOXO3a expression (Figure 3B).

### Measurement of Blood Pressure in All Groups of Pregnant Rats

Blood pressure of pregnant rats before and after hypertensive model preparation was shown in Figure 4. At gestation day 12, no significant difference of systolic pressure, diastolic pressure and average arterial pressure among all groups of pregnant rats were shown. At gestation day 16, comparing to normal pregnant group, systolic pressure, diastolic press and averaged arterial pressure were significantly up-regulated in L-NAME or L-NAME plus miR-155 inhibitor treated pregnant hypertensive rats, but not between L-NAME and L-NAME plus miR-155 inhibitor treated groups. At gestation day 20, significantly elevated systolic pressure, diastolic pressure and average arterial pressure were also found in L-NAME treated pregnant hypertensive group and L-NAME plus miR-155 inhibitor treated group compared to normal pregnant group ($p<0.05$). However, blood pressure in L-NAME + miR-155 inhibitor treated pregnant rats was reduced. Blood pressure in L-NAME plus si-FOXO3a group was significantly increased at gestation day 16 and day 20 compared to L-NAME group ($p<0.05$). These results suggested the protective role of FOXO3a in pregnant hypertension. Moreover, no significant difference of blood pressure appeared in normal pregnant rats among gestation day 12, day 16 and day 20. These results indicated that after miR-155 expression was reduced by inhibitor, blood pressure of pregnant rats can be effectively depressed.

### 24 h Urine Protein Assay

Twenty four hours total urine protein level was shown in Table I. At gestation day 12, no significant difference was found in paired comparison of 24 h urine protein among all groups. However, starting from gestation day 16, urine protein level in L-NAME and L-NAME + miR-155 inhibitor treated

**Table I.** Comparison of 24 h urine protein among all groups of pregnant mice (mg/24 h, mean±standard deviation).

| Group | N | Day 12 | Day 16 | Day 20 |
|---|---|---|---|---|
| Normal pregnant | 10 | 5.75±1.46 | 7.79±1.19 | 6.77±1.06 |
| L-NAME | 10 | 5.69±2.10 | 8.68±1.27 | 18.46±1.78** |
| L-NAME + miR-155 inhibitor | 10 | 5.81±1.78 | 8.89±1.03 | 12.31±2.18**## |
| L-NAME + si FOXO3a | 10 | 5.73±1.88 | 9.25±2.15 | 22.45±2.51**## |

**, $p<0.05$ comparing to normal pregnant group at the same time point; ##, p<0.05 comparing to L-NAME group at the same time.

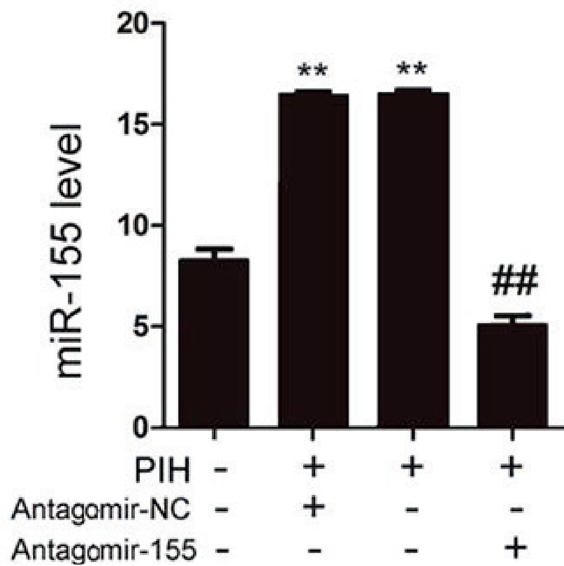



**Figure 1.** MiR-155 level assay. *, $p<0.05$ comparing to NC group. ##, $p<0.05$ comparing to pregnancy-induced hypertension (PIH) group.

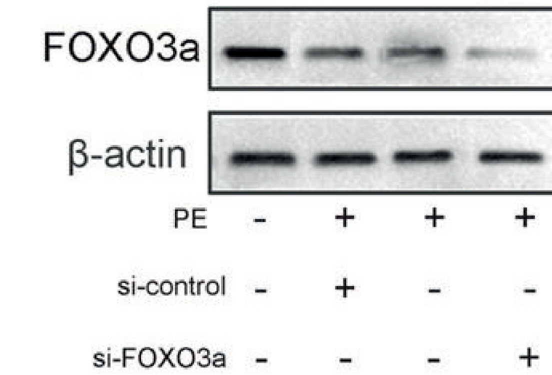



**Figure 2.** FOXO3a protein expression level. PIH, pregnancy-induced hypertension.

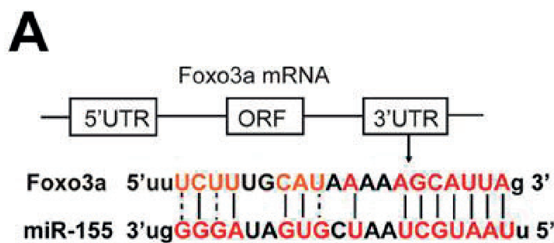
pregnant rat was increased to a certain extent, but showed no significant difference with normal pregnant group during the same period. At gestation day 10, comparing to normal pregnant group, 24 h urine protein level in L-NAME group was remarkably elevated ($p<0.05$). After using miR-155 inhibitor, 24 h urine protein level was significantly decreased, compared with L-NAME group ($p<0.05$).

### Placenta HE Staining of All Pregnant Rats

By HE staining on placental tissues, rat placenta can be divided into decidua layer, spongiotrophoblast layer, giant cell trophoblast layer and labyrinth trophoblast layer. No significant difference was observed in spongiotrophoblast layer of placenta tissues. Within labyrinth trophoblast layer, blood-tissue membrane works as the barrier for maternal fetal blood exchange. In normal pregnant rats, the blood cleft was wider, with regular arrangement of placental cells. By contrast, in L-NAME group, the blood cleft became thinner, accompanied with placental cell edema. MiR-155 inhibitor treatment improved cell arrangement and edema condition to certain extents. Si-FOXO3 treatment further deceased maternal blood cleft comparing to L-NAME group, with severe cell edema (Figure 5).

### Serum Creatinine Level

Assay for creatinine among all groups of pregnant rats was shown in Table II. At gestation day 12 and day 16, creatinine level among all groups presented no significant difference. But for results at gestation day 20, significantly higher serum creatinine level in L-NAME rats were observed compared with normal pregnant groups ($p<0.05$).



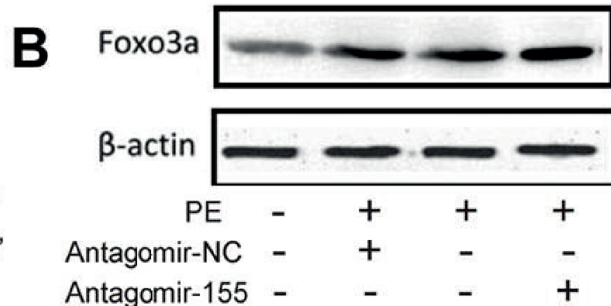




**Figure 3.** MiR-155 targeted regulation on FOXO3a expression. ***(A)*** Possible interaction between FOXO3a and miR-155; ***(B)*** MiR-155 inhibition up-regulates FOXO3a expression.

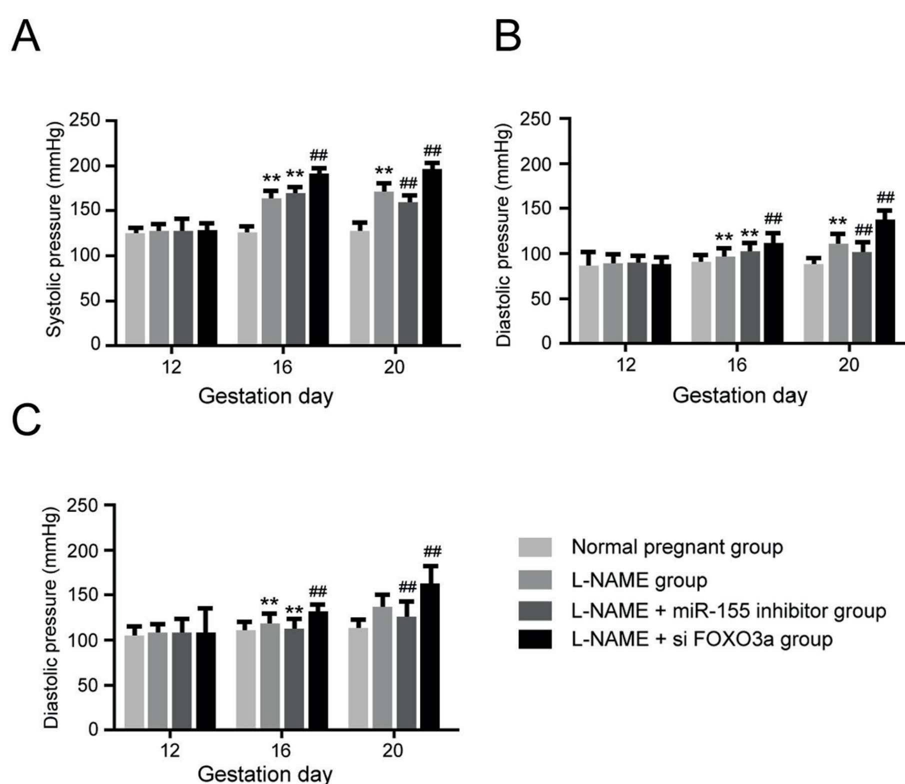



**Figure 4.** Blood pressure of pregnant rats. **, $p<0.05$ comparing to normal pregnant group. ##, $p<0.05$ comparing to L-NAME group.

**Table II.** Creatinine level of pregnant rats (μmol/L, mean±standard deviation).

| Group | N | Day 12 | Day 16 | Day 20 |
|---|---|---|---|---|
| Normal pregnant | 10 | 27.38±3.12 | 27.79±2.15 | 26.77±2.06 |
| L-NAME | 10 | 25.18±2.66 | 28.68±2.27 | 32.46±0.78** |
| L-NAME + miR-155 inhibitor | 10 | 25.81±2.78 | 28.89±2.03 | 28.19±2.18**## |
| L-NAME + si FOXO3a | 10 | 27.73±3.88 | 29.25±2.15 | 40.55±3.82**## |

**, $p<0.05$ comparing to Day 12; ##, $p<0.05$ comparing to Day 16.

Comparing to L-NAME group, serum creatinine level was decreased to certain extents in L-NMAE + miR-155 inhibitor group, whilst serum creatinine level in L-NAME + si-FOXO3a group was significantly elevated ($p<0.05$).

## Discussion

Pregnant hypertension is a type of specific disease in pregnant women which severely threatens maternal and fetal health. It has become one major reason causing death in pregnant women and neonatal[12]. Current drugs can be used to manage blood pressure, but the applications are frequently restricted during pregnancy due to various adverse effects. The pathogenesis mechanism of pregnant hypertension is still unclear, and most studies focus on the hormonal level change[13,14]. Therefore, the investigation of the role of miRNA in pregnant hypertension and potential treatment target is of critical importance for clinical treatment. NO is produced by vascular endothelial cells, and can regular blood vessel tension, dilate vessel, thus modulating cardiovascular system during pregnancy. As an inhibitor for NO, L-NAME can inhibit NO synthesis[15]. This work thus used L-NAME to establish pre-

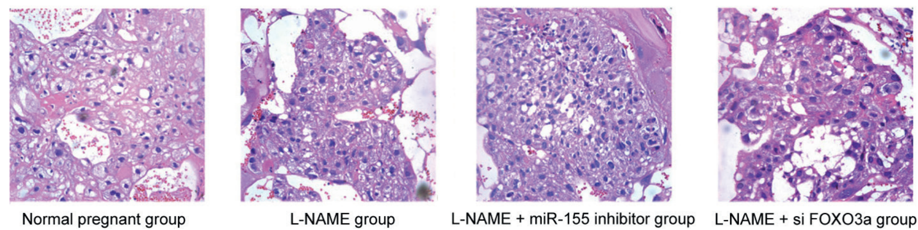



**Figure 5.** HE staining of pregnant rats. (Magnification: 40×).

gnant hypertension model, which was consistent with the central role of vascular endothelial cell damage during pregnant hypertension, and is widely accepted by scholars to mimic pregnant hypertension in clinics[16]. Recent investigations showed the involvement of certain miRNA molecules in blood pressure regulation. For example, miR-140-5p is remarkably down-regulated in pulmonary arterial hypertension[17]. Evidence also exhibited that miR-143[18] and miR-204[19] participated in hypertension regulation, but with little knowledge about related mechanism. These results indicated the potency of miRNA in blood pressure regulation. Pregnant hypertension is manifested as hypertension, edema and proteinuria. Previous studies[20] suggested the participation of miR-155 in occurrence of eclampsia. Recent findings reported up-regulation of miR-155 in early phase of eclampsia, and regulatory effects on endothelial cells by aspirin were correlated with suppressed miR-155/eNOS pathway[21]. In this work, we firstly generated a rat pregnant hypertension model, on which miR-155 expression was found to be significantly up-regulated, consistently with previous results. According to the database of Target Scan and miRna, we found that miR-155 might regulate FOXO3a[22,23], which was correlated with blood pressure regulation[24]. So, whether FOXO3a is involved in occurrence of pregnant hypertension has drawn our interests. In a rat pregnant hypertension model, we identified down-regulation of miR-155, accompanied with elevated blood pressure and urine protein level, which suggested the involvement of FOXO3a in pathology of pregnant hypertension. Consequently, we used FOXO3a siRNA to substantiate our conclusions. Meanwhile, using miR-155 inhibitor, we confirmed the regulatory role of miR-155 on FOXO3a. Placental development includes complicated process. The exchange of nutrient between fetus and maternal body is mainly achieved via blood exchange at maternal blood cleft of labyrinth trophoblast layer with fetal vessels[25]. In this study, from L-NAME group, thickening of fetal blood cleft, weakening nutrient exchange potency between maternal and fetal body were found, the fetal development of which was affected. MiR-155 inhibitor intervention alleviated this change to certain extents, thus facilitating fetal development. FOXO3a siRNA function further aggravates damage. These results demonstrated critical roles of miR-155 modulated FOXO3a expression in pregnant hypertension pathogenesis. However, how does miR-155 participate in regulating pregnant hypertension via targeting FOXO3a, and whether such pathway is dependent on NO related signal still require more delicate cell investigation.

## Conclusions

We found that miR-155 participates in regulating pregnant hypertension via targeting FOXO3a. Down-regulation of miR-155 level may serve as a therapeutic mean for treating pregnant hypertension.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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