Progesterone Enhances the Invasion of Trophoblast Cells by Activating PI3K/AKT Signaling Pathway to Prevent Preeclampsia

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Abstract

We aimed to explore whether the effect of progesterone on preeclampsia via the PI3K/AKT signaling pathway. First, we studied the role of progesterone in preeclampsia patients and HTR-8/Svneo cells by adding progesterone. Then PI3K inhibitor LY294002 was added. The effects of progesterone on preeclampsia were also studied in animals by constructing a preeclampsia rat model. CCK-8 and Transwell assay were applied to measure cell viability and invasion ability. ELISA was performed to measure progesterone, MMP-2, MMP-9, pro-inflammatory factors TNF-α, IL-1β, and anti-inflammatory factors IL-4, IL-10, and IL-13 levels. HE staining was used to detect the pathological changes in uterine spiral artery. Western blot was performed to detect Cyclin D1, PCNA, MMP-2, MMP-9, inflammatory factors TNF-α, IL-1β, IL-4, IL-10, IL-13, and PI3K/AKT signaling pathway related proteins AKT, p-AKT, PI3K, and p-PI3K expressions. Progesterone could reduce blood pressure and urine protein in pregnant women with preeclampsia. TNF-α and IL-1β levels were decreased, but IL-4, IL-10, IL-13, cyclin D1, and PCNA levels were increased in pregnant women with preeclampsia after using progesterone. After the use of progesterone, the symptoms of the PE model group were improved. Among them, the lumen of the placental uterine spiral artery was enlarged, and the fibrinoid necrosis of the uterine wall and acute atherosclerotic lesions were relieved. In addition, progesterone promoted HTR-8/Svneo cells proliferation and invasion. However, high expression of MMP-2, MMP-9, p-AKT, and p-PI3K in Normal and preeclampsia groups caused by progesterone was weakened after adding LY294002, indicating that progesterone could activate PI3K/AKT signaling pathway to regulate HTR-8/Svneo cells. Progesterone decreased urine protein and blood pressure of preeclampsia rats in a concentration-dependent manner. Moreover, progesterone activated the PI3K/AKT signaling pathway and inhibited the inflammatory response in preeclampsia rats.

Keywords

Introduction

Preeclampsia was a serious pregnancy-specific disease characterized by hypertension and proteinuria in the 20th week of pregnancy[1,2]. It was considered the leading cause of perinatal morbidity and the mortality of maternal and infants[3]. Preeclampsia risk factors include obesity, insulin resistance, and hyperlipidemia, which can stimulate the release of inflammatory and oxidative stress, leading to endothelial dysfunction[4]. In preeclampsia, there was abnormal invasion of trophoblast cells and destruction of the physiological remodeling of the uterine spiral artery into uterine placental vessels observed in normal pregnancy[5,6]. Severe preeclampsia may be associated with thrombocytopenia, multiple organ damage, and fetal growth restriction[7]. However, the pathologic mechanisms are not fully understood. Therefore, an in-depth understanding of the pathogenesis of preeclampsia is critical to providing more effective treatment for preeclampsia.

Progesterone is important for endometrial implantation, differentiation, and placenta formation. Progesterone stimulates

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uterine growth and opposes uterine contractions. The reduction of progesterone will cause the weight of the fetus and placenta to decrease[8–10]. However, the expression and role of progesterone in preeclampsia are controversial. It was reported that the serum progesterone level of preeclampsia patients has begun to rise before the onset of clinical manifestations compared with normal pregnancy. Then progesterone levels are always higher than normal pregnant women with the development of preeclampsia[11,12]. You et al.[13] found that while inhibiting vasoconstriction, progesterone could promote vasodilation through nitric oxide and increase placental perfusion. Hu et al. reported that progesterone could modulate the activity of calcium channels in uterine arteries. Insufficient progesterone secretion will lead to the overload of intracellular calcium ions, induce vascular smooth muscle contraction, vascular stenosis, and insufficient blood supply, which may be the initial factor for the onset of preeclampsia[14]. However, Sammour et al.[15] have believed that progesterone may be a new way to treat or prevent preeclampsia. In addition, in a pregnant rat model, 17-hydroxyprogesterone could reduce hypertension induced by TNF-α[16]. Therefore, we wanted to explore the effects of progesterone on preeclampsia and the mechanisms involved.

Recently, more and more evidence has shown the PI3K/AKT signaling pathway was related to the pathogenesis of preeclampsia. The literature found that the PI3K/AKT signaling transduction was involved in regulating trophoblast cells[17,18]. Activating the PI3K/AKT signaling pathway in placenta of preeclampsia could regulate cell proliferation[19]. The activation of the PI3K/AKT/eNOS signaling pathway was vital in regulating cell migration, angiogenesis, and preventing hypertension[20]. Studies have shown that the PI3K/AKT signaling pathway was involved in the response of progesterone to regulate sperm motility[21]. The PI3K/AKT signaling pathway might be involved in the down-regulation of PDCD4 protein induced by progesterone[22]. However, there are few studies on the involvement of progesterone in the PI3K/AKT signaling pathway for preeclampsia.

Therefore, we mainly wanted to investigate whether progesterone acts on trophoblast cells via the PI3K/AKT signaling pathway in the pathogenesis of preeclampsia, which might provide a new therapeutic target for treating preeclampsia, thus providing reference and basis for the prevention and clinical treatment of preeclampsia.

Materials and Methods

Clinical Samples

Forty participants were from the Second Xiangya Hospital from January 2020 to July 2020. They were divided into normal pregnant women without progesterone (Normal), normal pregnant women with progesterone (Normal+progesterone), pregnant women with preeclampsia without progesterone (preeclampsia), and pregnant women with preeclampsia with progesterone (preeclampsia+progesterone). The inclusion criteria of this study were as follows: age 26-40 years old, gestational weeks 32-39 weeks, singleton, no previous diabetes, heart disease, hypertension, kidney disease and thyroid disease, no fetal abortion, stillbirth, spontaneous abortion, and so on. No history of adverse pregnancy and childbirth, no history of assisted reproduction, smoking, drinking, and no other drugs. Preeclampsia is diagnosed in pregnant women who meet the following diagnostic criteria[23]: after 20 weeks of gestation, systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥110 mmHg, random urine protein (+), or not accompanied by proteinuria, but any of the following other manifestations: thrombocytopenia, blood routine indicates platelets $<100 \times 10^9$/L; abnormal liver function, mainly manifested as serum transaminase levels that are two times or more the normal value; renal function damage, manifested as serum creatinine value > 1.1 mg/dL or more than two times the normal value; no previous pulmonary abnormality, but now pulmonary edema; no previous central nervous system abnormality or visual disorder, but now new onset symptom. The exclusion criteria of the subjects in this study were as follows: hypertension that had been discovered before 20 weeks, chronic hypertension; concurrent infection, malignant tumor, severe heart, liver and kidney disease, autoimmune system disease, endocrine system disease and other acute and chronic medical and surgical diseases; combined with other obstetric complications other than preeclampsia, such as placenta previa, gestational diabetes mellitus, placental abruption; multiple pregnancy; assisted reproductive conception; labor before cesarean section or vaginal delivery; previous smoker alcohol history, oral progesterone, intramuscular progesterone, oral euthyrox, immunosuppressive drugs and other drugs; pregnant women's obstetric examination data are incomplete. Normal+progesterone group and preeclampsia+progesterone group women took progesterone 60 mg Bid orally from conception until 12 weeks of pregnancy. Blood pressure before abortion was observed. Progesterone, pro-inflammatory factors (TNF-α and IL-1β), and anti-inflammatory factors (IL-4, IL-10, and IL-13) were measured in serum and placenta tissues. Placenta tissue morphology was observed. The study was agreed by all participants and the ethics number was LYF2021065. The basic information on subjects is shown in Table 1. The CONSORT flow chart is shown in Figure S1.

Cell Culture and Treatment

HTR-8/SVneo trophoblast cells were purchased from the Cell Bank of the Chinese Academy of Sciences, cultured in RPMI-1640 medium containing 10% FBS (Thermo Fisher Scientific), and placed at 37°C, cultivated in a humified chamber with 5% CO_2. HTR-8/SVneo cells were cultured in Normal pregnant women or pregnant women with preeclampsia serum. To study the effect of progesterone concentration on HTR-8/SVneo cells of normal pregnant women or pregnant women with preeclampsia[24], we set low, medium

Table 1. The Basic Information of Subjects.

Group	n	Age	Average age	Diagnosis	Patients with severe symptoms	Whether to use progesterone	Blood pressure	Urine protein	Neonatal weight
Normal	10	26-40	30.15±4.30	intrauterine pregnancy	0%	No	90-140/60-90 mmHg	−	3000-3800 g
Normal+progesterone	10	26-40	29.18±3.53	intrauterine pregnancy	0%	Yes (60 mg Bid progesterone)	90-140/60-90 mmHg	−	3000-3800 g
Preeclampsia	10	26-40	28.25±2.60	Preeclampsia	0%	No	130-180/80-140 mmHg	+	1000-3000 g
Preeclampsia +progesterone	10	26-40	30.43±3.50	Preeclampsia	0%	Yes (60 mg Bid progesterone)	130-180/80-140 mmHg	+	1000-3400 g

and high concentrations progesterone (10^{-8}mol/L, 10^{-6}mol/L, 10^{-4}mol/L) and grouped them into the Normal, Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone, preeclampsia, preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, preeclampsia +10^{-4}mol/L progesterone groups. Then we chose 10^{-4}mol/L progesterone for follow-up experiments, grouped them into the Normal, preeclampsia, Normal+progesterone, preeclampsia+progesterone groups. In all, 5 μM PI3K inhibitor LY294002 was added to investigate whether progesterone acted on HTR-8/SVneo cells through the PI3K/AKT signaling pathway. They were divided into the Normal, Normal+LY, preeclampsia, preeclampsia+LY, Normal+ progesterone, Normal+LY+progesterone, preeclampsia+ progesterone, and preeclampsia+LY+progesterone groups.

Animals

SPF Wistar rats were used in the study. The animal protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) (No.:2020845).

Protocol 1: To confirm that the administration of L-NAME established the preeclampsia model. The 64 Wistar female rats were randomly divided into 2 groups: normal pregnant rats without progesterone (Normal) and preeclampsia rats without progesterone (preeclampsia). From the 12th day of gestation, pregnant mice in the preeclampsia group were subcutaneously injected with nitric oxide synthase inhibitor N^G-Nitro-L-arginine Methyl Ester (L-NAME, 125 mg/kg/d) for 7 days. The pregnant mice in the Normal group were subcutaneously injected with normal saline (the same volume as L-NAME).

Protocol 2: To confirm the effect of different concentrations of progesterone on preeclampsia. The 64 Wistar rats were randomly divided into the Normal, Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone, preeclampsia, preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, and preeclampsia+10^{-4}mol/L progesterone groups, with 8 rats in each group. Progesterone was dissolved into sesame oil at a concentration of 12 mg/mL, and then 8 mg/mL and 4 mg/mL were prepared with sesame oil. Normal pregnant rats and preeclampsia rats were given daily oral progesterone

(0 mol/L, 10^{-8}mol/L, 10^{-6}mol/L, 10^{-4}mol/L) starting from 9th day of gestation. The changes in blood pressure and urine protein concentration in 0–20 days were observed and recorded every three days. After 20 days, progesterone, pro-inflammatory factors (TNF-α and IL-1β), and anti-inflammatory factors (IL-4, IL-10, and IL-13) were measured in serum and placenta tissues. Placenta tissue morphology was observed.

Measurement of Systolic Blood Pressure and Urine Protein Quantitation

A tail cuff and a pneumatic pulse transducer (BP-98A, Softron, Japan) were used to monitor the systolic blood pressure (mmHg) of rats in each group every morning. BCA protein assay kit (Beyotime, Beijing, China) was used to measure the urine protein of each group at 0, 3, 6, 9, 12, 15, and 18 days.

Hematoxylin-Eosin (HE) Staining

Placenta tissues were fixed with 10% formaldehyde for 24 h, embedded in conventional paraffin, and sectioned (thickness 4-5 μm), stained with HE, dehydrated, and sealed with neutral resin. The diameter changes of the placental uterine spiral artery were detected.

Cell Counting Kit 8 (CCK-8) Assay

HTR-8/SVneo cells at the logarithmic growth stage were inoculated in 96-well plates with 1×10^4 cells/well, incubated at 37°C and 5% CO_2 for 12 h. After 24 h treatment, 10 μL CCK-8 reagent/well was added to the incubator for 2 h. Then we measured the absorbance at 450 nm wavelength using a Microplate Reader (Infinite M200, Tecan, Austria), and cell viability (12, 24, 48, and 72 h) was assessed for each group.

Transwell Assay

HTR-8/Svneo cells were collected using the transwell system, resuspended with serum-free DMEM, and inoculated into the upper chamber of the transwell plate. Matrigel glue was dissolved at 4°C overnight, and diluted with a pre-cooled basal medium at a ratio of 1:3 (matrigel: medium). Then we added 40 μL of matrigel glue to the pre-cooled transwell chamber and incubated at 37°C for 2 h to solidify matrigel

Table 2. The Basic Information About ELISA Kits.

Name	Article number	Detection range	Sensitivity	Company
Human Progesterone	CSB-E07283h	0.15 ng/mL-70 ng/mL	0.2 ng/mL	CUSABIO (China)
Rat Progesterone	CSB-E07282r	0.15 ng/mL-70 ng/mL	0.2 ng/mL	CUSABIO (China)
Human MMP-2	CSB-E04675h	0.78 ng/mL-50 ng/mL	0.195 ng/mL	CUSABIO (China)
Rat MMP-2	CSB-E07411r	0.625 ng/mL-40 ng/mL	0.156 ng/mL	CUSABIO (China)
Human MMP-9	CSB-E08006h	0.312 ng/mL-20 ng/mL	0.284 ng/mL	CUSABIO (China)
Rat MMP-9	CSB-E08008r	0.78 ng/mL-50 ng/mL	0.195 ng/mL	CUSABIO (China)
Human TNF-α	CSB-E04740h	7.8 pg/mL-500 pg/mL	1.95 pg/mL	CUSABIO (China)
Rat TNF-α	CSB-E11987r	6.25 pg/mL-400 pg/mL	1.56 pg/mL	CUSABIO (China)
Human IL-1β	CSB-E08053h	7.8 pg/mL-500 pg/mL	1.95 pg/mL	CUSABIO (China)
Rat IL-1β	CSB-E08055r	0.156 ng/mL-10 ng/mL	0.039 ng/mL	CUSABIO (China)
Human IL-4	CSB-E04633h	31.25 pg/mL-2000 pg/mL	7.8 pg/mL	CUSABIO (China)
Rat IL-4	CSB-E04635r	15.6 pg/mL-1000 pg/mL	3.9 pg/mL	CUSABIO (China)
Human IL-10	CSB-E04593h	31.25 pg/mL-2000 pg/mL	7.8 pg/mL	CUSABIO (China)
Rat IL-10	CSB-E04595r	62.5 pg/mL-4000 pg/mL	15.6 pg/mL	CUSABIO (China)
Human IL-13	CSB-E04601h	0.39 ng/mL-25 ng/mL	0.09 ng/mL	CUSABIO (China)
Rat IL-13	CSB-E07454r	7.8 pg/mL-500 pg/mL	1.95 pg/mL	CUSABIO (China)

ELISA: Enzyme-Linked Immunosorbent Assay; IL: interleukin; TNF: tumor necrosis factor; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9.

glue and counted cells with basic medium and adjusted to 1×10^6/mL. Then we added 100 μL cells to the upper chamber and 600 μL of complete medium to lower chamber. After incubation at 37°C for 24 h, the cells on the upper chamber surface were wiped with wet cotton swabs, fixed with 4% paraformaldehyde for 20 min, stained with 0.5% crystal violet for 5-10 min, and observed and photographed under the microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

Progesterone, MMP-9, pro-inflammatory factors (TNF-α and IL-1β) and anti-inflammatory factors (IL-4, IL-10, and IL-13) were detected by ELISA Kit according to the manufacturer's instructions. The basic information about these kits is shown in Table 2. The samples were in triplicate. The DYNATECHMR7000 enzyme-labeled instrument (USA) was used to monitor the absorbance values of each group. The concentrations of progesterone, MMP-2, MMP-9, TNF-α, IL-1β, IL-4, IL-10, and IL-13 were calculated through the standard curve.

Western Blot

RIPA lysis buffer (#P0013B, Beyotime, China) was used to extract the total protein from tissues and cells. The protein of each group was quantified according to the BCA protein determination kit. The protein was mixed with SDS-PAGE loading buffer (#MB2479, Meilunbio, China) for 5 min in boiling water at 100°C. The protein was adsorbed on the PVDF membrane by gel electrophoresis and sealed with 5% skim milk solution for 2 h at room temperature, and incubated with diluted primary antibodies including TNF-α (17590-1-AP, 1:1000, proteintech, USA), IL-1β (16806-1-AP, 1:1000, proteintech, USA), IL-4 (66142-1-Ig, 1:1000,

proteintech, USA), IL-10 (20850-1-AP, 1:1000, proteintech, USA), IL-13 (ab106732, 1:1000, abcam, UK), cyclin D1 (60186-1-Ig, 1:6000, proteintech, USA), PCNA (10205-2-AP, 1:4000, proteintech, USA), MMP-2 (10373-2-AP, 1:1000, proteintech, USA), MMP-9 (10375-2-AP, 1:1000, proteintech, USA), AKT (10176-2-AP, 1:3000, proteintech, USA), p-AKT (66444-1-Ig, 1:3000, proteintech, USA), PI3K (ab40755, 1:2000, proteintech, USA), p-PI3K (ab182651, 1:1000, abcam, UK) and β-actin (66009-1-Ig, 1:1000, abcam, UK) at 4°C overnight. The secondary antibody HRP-goat anti-mouse IgG (SA00001-1, 1:5000, proteintech, USA) or HRP-goat anti-rabbit IgG (SA00001-2, 1:6000, proteintech, USA) was incubated with the membrane at room temperature for 90 min. The protein bands were detected by the Chemiscope6100 system (Clinx Co., Ltd, Shanghai, China). β-actin was used as an internal reference for detecting relative expression levels.

Statistical Analysis

Graphpad 8.0 was used for statistical analysis, and experimental data were expressed as mean $\pm$ standard deviation (SD), which was repeated at least 3 times. Student's t test was used between the two groups, and one-way analysis of variance (ANOVA) was used for comparison between multiple groups. $P < 0.05$ was considered statistically significant.

Results

Progesterone Supplementation Improved Blood Pressure, Progesterone Levels, and Inflammation in Pregnant Women With Preeclampsia

To study the effect of progesterone on normal pregnant women and pregnant women with preeclampsia, we first
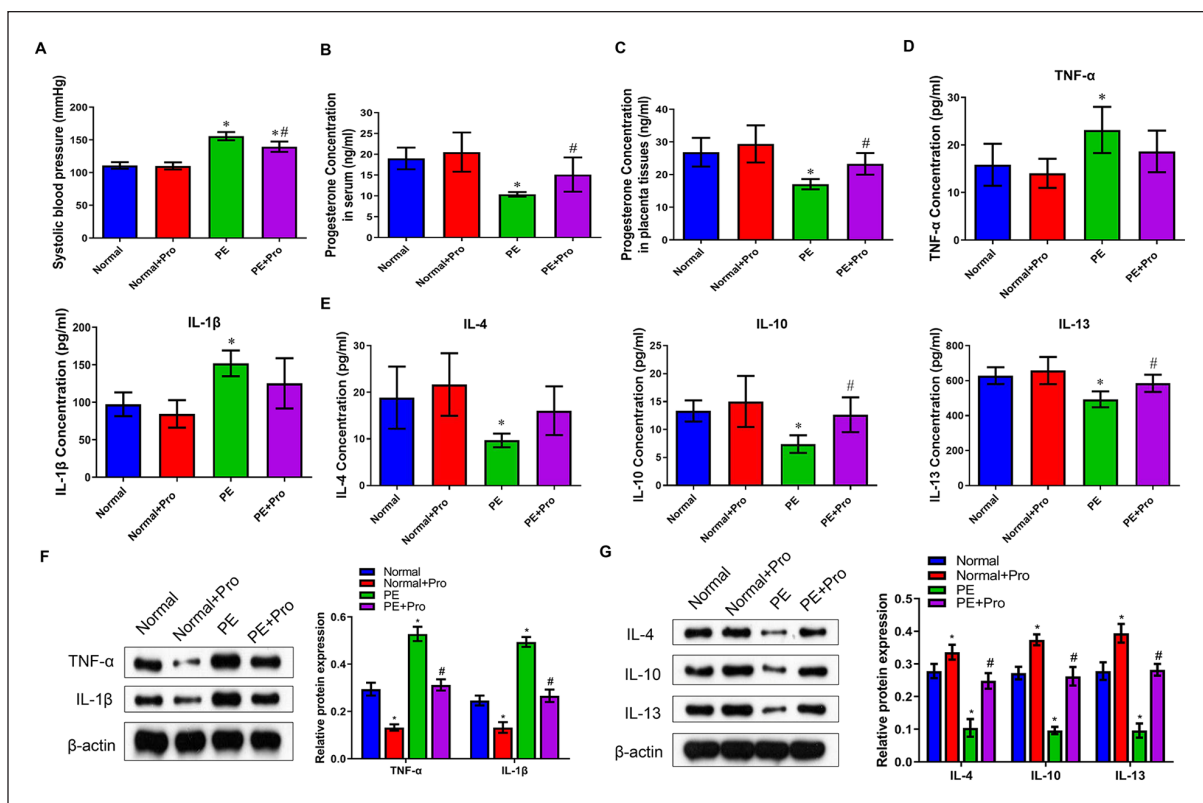
Figure 1. Progesterone supplementation improved blood pressure, progesterone levels, and inflammation in pregnant women with preeclampsia. In the Normal, Normal+ progesterone, preeclampsia, preeclampsia+ progesterone groups. (A) Systolic blood pressure before the abortion. (B) The level of progesterone in serum. (C) The level of progesterone in placenta tissues. (D) Serum pro-inflammatory factors TNF-α and IL-1β levels. (E) Serum anti-inflammatory factors IL-4, IL-10, and IL-13 levels. (F) The pro-inflammatory factors TNF-α and IL-1β expression in placenta tissues. (G) The anti-inflammatory factors IL-4, IL-10, and IL-13 expression in placenta tissues. Pro: progesterone; PE: preeclampsia; IL: interleukin; TNF: tumor necrosis factor. *P <0.05 vs Normal group; #P <0.05 vs preeclampsia group.

performed systolic blood pressure testing. Compared with the Normal group, the systolic blood pressure of the preeclampsia group increased significantly, but after using progesterone, the systolic blood pressure decreased (Fig. 1A). ELISA results showed that progesterone levels in serum and placenta tissues of the preeclampsia group were significantly lower than the Normal group, while progesterone content increased after using progesterone (Fig. 1B, C). Then ELISA was performed to detect serum pro-inflammatory factors TNF-α, IL-1β, and anti-inflammatory factors IL-4, IL-10, and IL-13 levels. Compared with the Normal group, pro-inflammatory factors TNF-α and IL-1β levels in the preeclampsia group increased significantly, but anti-inflammatory factors IL-4, IL-10, and IL-13 levels decreased significantly. After using progesterone, pro-inflammatory TNF-α and IL-1β levels decreased, but anti-inflammatory factors IL-4, IL-10, and IL-13 levels increased (Fig. 1D, E). Western blot results were consistent with the ELISA results (Fig. 1F, G). These results indicated that progesterone could improve blood pressure and inflammation in pregnant women with preeclampsia.

Progesterone Caused Histological Changes in the Placenta, Affected Cell Proliferation and Invasion, and PI3K/AKT Signaling Pathway

HE staining showed that placental uterine spiral arteries in the Normal group showed physiological changes during pregnancy, with the enlarged lumen and thinner walls. The placental uterine spiral arteries lacked such physiological changes in the preeclampsia group. The placental uterine spiral arteries were mostly seen with thickening of the wall, luminal stenosis, fibrinoid necrosis and acute atherosclerotic lesions. After progesterone treatment in the preeclampsia+progesterone group, the lumen of the placental uterine spiral artery was enlarged, and the fibrinoid necrosis of the uterine wall and acute atherosclerotic lesions were relieved (Fig. 2A). To explore whether progesterone affected trophoblast cell proliferation and invasion and the PI3K/AKT signaling pathway, Western blot was used to detect cyclin D1, PCNA, MMP-2, MMP-9, AKT, and p-AKT expression in placenta tissues. The results showed that the expression of Cyclin D1, PCNA, MMP-2,
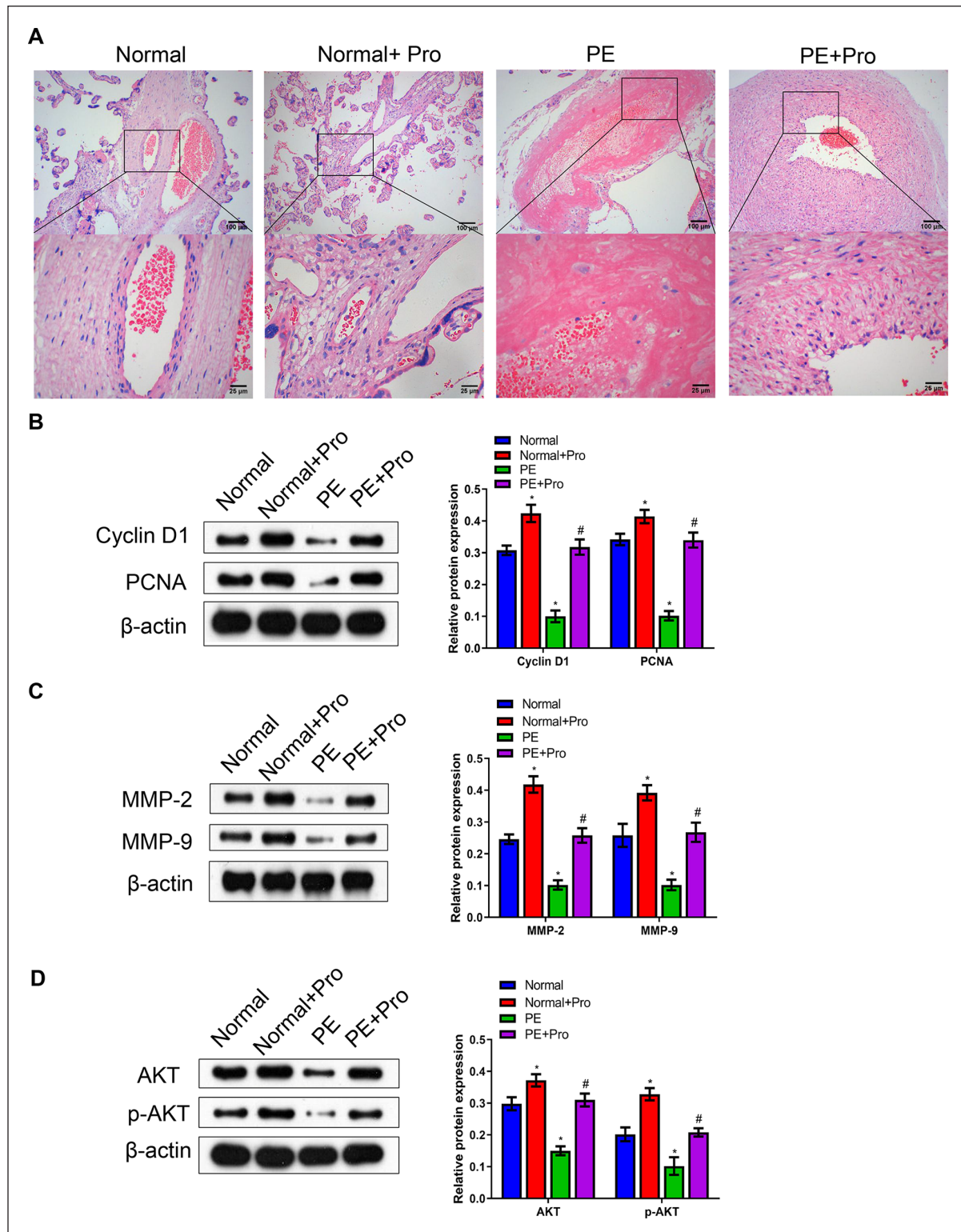
Figure 2. Progesterone caused histological changes in the placenta, affected cell proliferation and invasion, and the PI3K/AKT signaling pathway. In the Normal, Normal+ progesterone, preeclampsia, preeclampsia+ progesterone groups. (A) Morphological photograph of placenta tissues stained with HE. (B) Cyclin D1 and PCNA expression in placenta tissues. (C) MMP-2 and MMP-9 expression in placenta tissues. (D) AKT and p-AKT expression in placenta tissues. Scale bar = 100 μm; The magnification was 100 times and 400 times respectively; Pro: progesterone; PE: preeclampsia; HE: Hematoxylin-Eosin; PCNA: proliferating cell nuclear antigen; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; AKT: protein kinase B; p-AKT: phosphorylation of protein kinase B. *P <0.05 vs Normal group; #P <0.05 vs preeclampsia group.
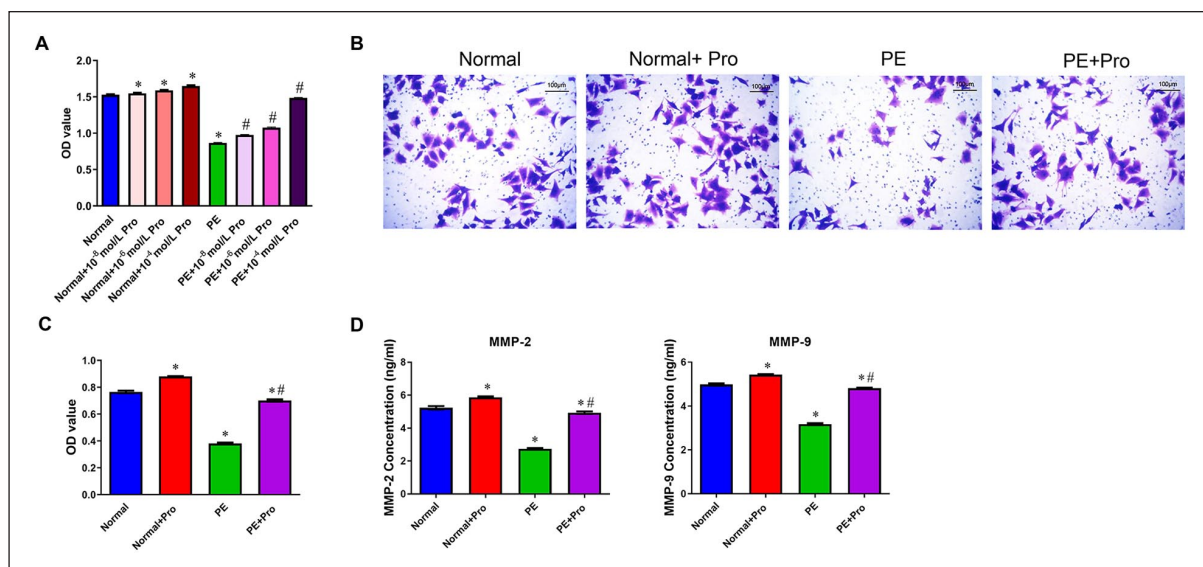
Figure 3. Progesterone promoted HTR-8/Svneo cell proliferation and invasion. (A) CCK-8 was performed to detect cell proliferation in the Normal, Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone, preeclampsia, preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, preeclampsia+10^{-4}mol/L progesterone groups. In the Normal, Normal+ progesterone, preeclampsia, preeclampsia+ progesterone groups. (B) Transwell assay was applied to measure cell invasion. (C) Quantitative detection of cell invasion rate. (D) MMP-2 and MMP-9 levels. Scale bar = 100 μm; The magnification was 100 times; Pro: progesterone; PE: preeclampsia; CCK-8: Cell Counting Kit 8; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; OD: optical density. *$P <0.05$ vs Normal group; #$P <0.05$ vs preeclampsia group.

MMP-9, AKT, and p-AkT in placenta tissues of the Normal+progesterone group were increased compared with the Normal group, but Cyclin D1, PCNA, MMP-2, MMP-9, AKT, and p-AKT expression of the preeclampsia group were significantly decreased. However, cyclin D1, PCNA, MMP-2, MMP-9, AKT, and p-AKT expression increased after adding progesterone (Fig. 2B–D). These results suggested that progesterone could promote cell proliferation in preeclampsia, which involved the PI3K/AKT signaling pathway.

Progesterone Promoted HTR-8/Svneo Cell Proliferation and Invasion

To explore the effect of progesterone on HTR-8/Svneo cells, CCK-8 was applied to detect cell proliferation. The results showed that the cell activity of Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone groups was increased compared with the Normal group, but that of the preeclampsia group was decreased. After the use of progesterone in preeclampsia, as the progesterone concentration increased, cell viability gradually increased (Fig. 3A). Transwell assay results showed that compared with the Normal group, HTR-8/SVneo cell invasion rate in the Normal+progesterone group increased, while the invasion rate of the preeclampsia group decreased. After using progesterone, the invasion rate of HTR-8/SVneo cells increased significantly (Fig. 3B, C). ELISA results showed that compared with the Normal group, MMP-2 and MMP-9 levels in the Normal+progesterone group increased, while

MMP-2 and MMP-9 levels in preeclampsia group decreased significantly. After using progesterone, MMP-2 and MMP-9 levels increased (Fig. 3D). The above results revealed that progesterone could promote HTR-8/SVneo cell proliferation and invasion.

Progesterone Acted on HTR-8/Svneo Cells via the PI3K/AKT Signaling Pathway

In order to study whether progesterone acted on trophoblast cells via the PI3K/AKT signaling pathway, PI3K inhibitor LY294002 was used. As shown in Fig. 4A, B, cell proliferation and invasion abilities decreased in preeclampsia group and Normal+LY group compared with Normal group. Meantime, cell proliferation and invasion abilities also inhibited in preeclampsia+LY group than that in preeclampsia group. After using progesterone, cell proliferation and invasion ability also decreased in Normal+LY+progesterone and preeclampsia+progesterone groups than Normal+progesterone group. After adding LY294002, cell proliferation and invasion abilities were notably decreased in the preeclampsia+LY+progesterone group than in preeclampsia+progesterone. ELISA showed that MMP-2 and MMP-9 levels in the preeclampsia group and Normal+ LY group were significantly decreased compared with the Normal group. Compared with the preeclampsia group, MMP-2 and MMP-9 levels in the preeclampsia+LY group were significantly reduced. After using progesterone, the levelsofMMP-2andMMP-9intheNormal+LY+progesterone

and preeclampsia+progesterone groups also decreased significantly compared with the Normal+progesterone group. Compared with the preeclampsia+progesterone group, MMP-2 and MMP-9 levels in the preeclampsia+LY+progesterone group were significantly reduced (Fig. 4C). Western blot was performed to detect AKT, p-AKT, PI3K, and p-PI3K expression. Results showed that the changes in AKT and PI3K were not significant. Compared with the Normal group, the expression of p-AKT and p-PI3K in Normal+LY and preeclampsia groups were significantly decreased. Compared with the preeclampsia group, the expression of p-AKT and p-PI3K in the preeclampsia+LY group were significantly reduced after adding PI3K inhibitor LY294002. The expression of p-AKT and p-PI3K in Normal+LY+progesterone and preeclampsia+ progesterone groups were also significantly decreased compared with the Normal+ progesterone group after using progesterone. Compared with the preeclampsia+progesterone group, p-AKT and p-PI3K expression in the preeclampsia+LY+progesterone group were significantly reduced after adding PI3K inhibitor LY294002 (Fig. 4D). The above results showed high expression of MMP-2, MMP-9, p-AKT, p-PI3K in Normal, and preeclampsia groups were weakened by the use of PI3K inhibitor LY294002, indicating that progesterone could activate the PI3K/AKT signaling pathway to regulate HTR-8/Svneo cells.

Effects of Different Concentrations of Progesterone on Blood Pressure, Urinary Protein, and Inflammatory Factors in Preeclampsia Rats

To further investigate the effect of progesterone on normal pregnant rats and preeclampsia rats, we set different concentrations of progesterone. First, by detecting blood pressure and urine protein, we found no significant difference in blood pressure between the eight groups on days 0-9 of pregnancy. With the number of days of pregnancy increased, there was no significant difference in blood pressure between the Normal and Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone groups. In contrast, the blood pressure of the preeclampsia group increased gradually. After the use of progesterone in preeclampsia, the blood pressure decreased significantly with the increase of progesterone concentration (Fig. 5A). There was no significant difference in urine protein between the Normal and Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone groups at 0-18 days of pregnancy, while urine protein in preeclampsia group increased gradually with the increase of pregnancy days. After the use of progesterone in preeclampsia, the decrease of urine protein was more significantly with the increase of progesterone concentration (Fig. 5B). To study the effect of progesterone on cell invasion, MMP-2, MMP-9, and progesterone levels in serum were detected by ELISA, and it was showed that MMP-2,

MMP-9, and progesterone levels were gradually increased in the Normal, Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, and Normal+10^{-4}mol/L progesterone groups at 0-18 days of gestation, as well as the preeclampsia, preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, and preeclampsia+10^{-4}mol/L progesterone groups. However, in general, MMP-2, MMP-9, and progesterone levels in the preeclampsia, preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, and preeclampsia+10^{-4}mol/L progesterone groups were lower than those in the Normal, Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, and Normal+10^{-4}mol/L progesterone groups. Compared with Normal and preeclampsia groups, the levels of MMP-2, MMP-9, and progesterone in the Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone groups and preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, and preeclampsia+10^{-4}mol/L progesterone groups increased with the increase of progesterone concentration (Fig. 5C, D).

To study the influence of different concentrations of progesterone on inflammation response, pro-inflammatory factors TNF-α, IL-1β and anti-inflammatory factors IL-4, IL-10, and IL-13 levels in serum were detected by ELISA. Pro-inflammatory factors TNF-α, IL-1β levels decreased slightly in the Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone groups were slightly decreased compared with the Normal group, but anti-inflammatory IL-4, IL-10, and IL-13 levels were slightly increased, but not significantly. In the preeclampsia group, pro-inflammatory factors TNF-α, IL-1β levels increased significantly, and anti-inflammatory factors IL-4, IL-10, IL-13 levels decreased significantly compared with the normal group. After the use of progesterone in preeclampsia, pro-inflammatory factors TNF-α and IL-1β levels gradually decreased, but anti-inflammatory factors IL-4, IL-10, and IL-13 levels gradually increased with the increase of progesterone concentration (Fig. 5E, F). These results suggested that progesterone could improve preeclampsia's blood pressure and urine protein, promote cell invasion, and improve inflammatory response. With the increase of progesterone concentration, its effect was more significant.

Different Concentrations of Progesterone Activated the PI3K/AKT Signaling Pathway, Promoted Cell Proliferation and Invasion of Placenta Tissues, and Inhibited the Inflammatory Response in Preeclampsia Rats

HE staining results showed that the placental uterine spiral arteries in the Normal group showed physiological changes during pregnancy, with the enlarged lumen and thinner walls. The placental uterine spiral arteries lacked such physiological changes in the preeclampsia group. The placental uterine
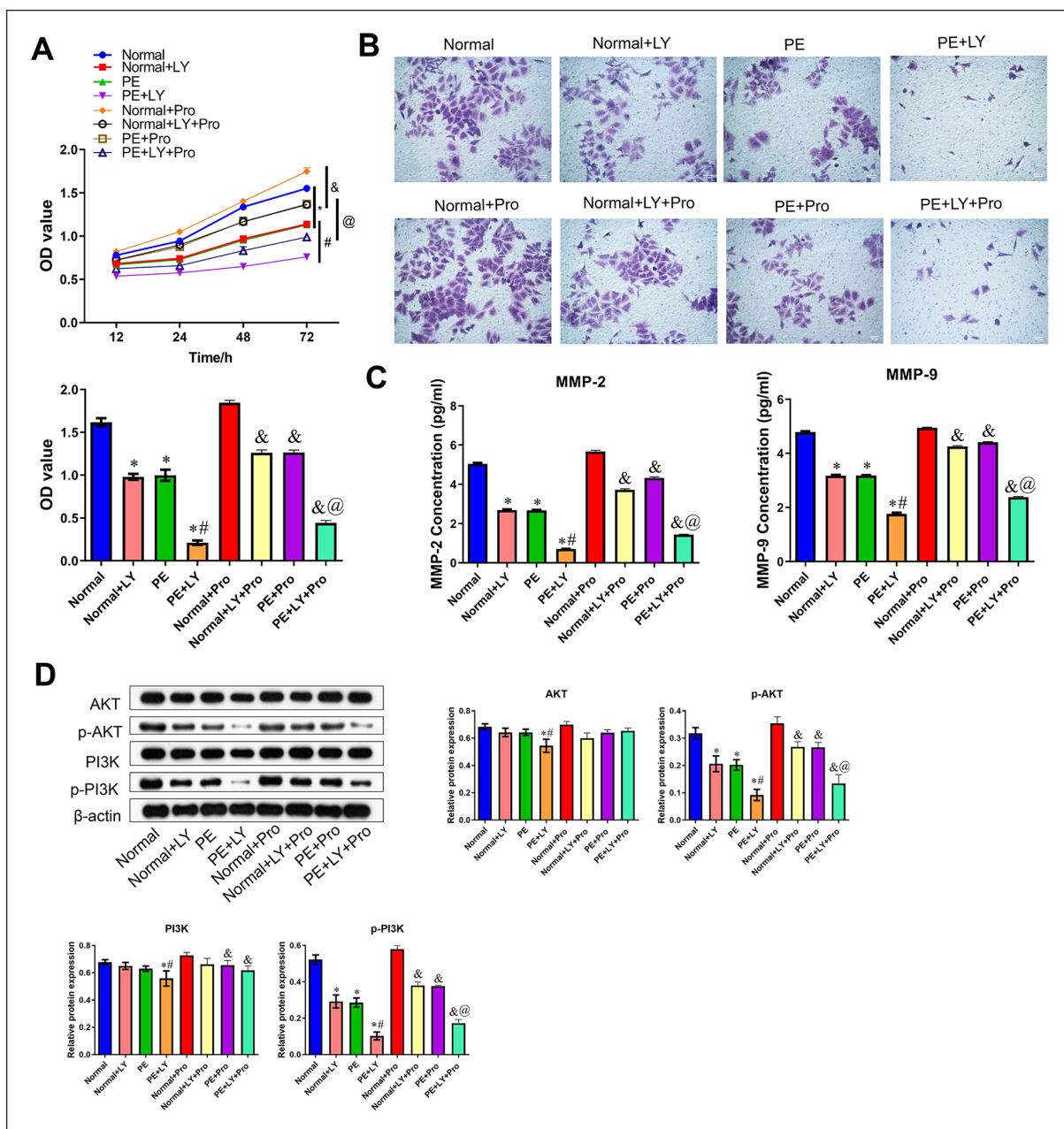
Figure 4. Progesterone acted on HTR-8/Svneo cells via the PI3K/AKT signaling pathway. In the Normal, Normal+LY, preeclampsia, preeclampsia+LY, Normal+ progesterone, Normal+LY+ progesterone, preeclampsia+ progesterone, preeclampsia+LY+ progesterone groups. (A) CCK-8 was performed to measure cell proliferation. (B) Transwell assay was applied to evaluate cell invasion. (C) MMP-2 and MMP-9 levels. (D) AKT, p-AKT, PI3K, and p-PI3K expressions. Pro: progesterone; PE: preeclampsia; CCK-8: Cell Counting Kit 8; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; OD: optical density; p-PI3K: phosphorylation of phosphoinositide 3-kinase; PI3K: phosphorylation of phosphoinositide 3-kinase; AKT: protein kinase B; p-AKT: phosphorylation of protein kinase B; LY: LY294002. *P <0.05 vs Normal group; #P <0.05 vs preeclampsia group; &P <0.05 vs Normal+progesterone group; @P <0.05 vs preeclampsia+progesterone group.

spiral arteries were mostly seen with thickening of the wall, and luminal stenosis. After the use of progesterone in pre-eclampsia, the placental uterine spiral artery could be seen thinner and larger lumen with the increase of progesterone concentration (Fig. 6A). To investigate the effects of different concentrations of progesterone on cell proliferation and invasion, Western blot was applied to detect Cyclin D1,

PCNA, MMP-2, and MMP-9 expressions in placenta tissues. Results showed that compared with the Normal group, cyclin D1, PCNA, MMP-2, and MMP-9 expression increased in the Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone groups, while the expression of cyclin D1, PCNA, MMP-2, and MMP-9 decreased in preeclampsia group. After the use of
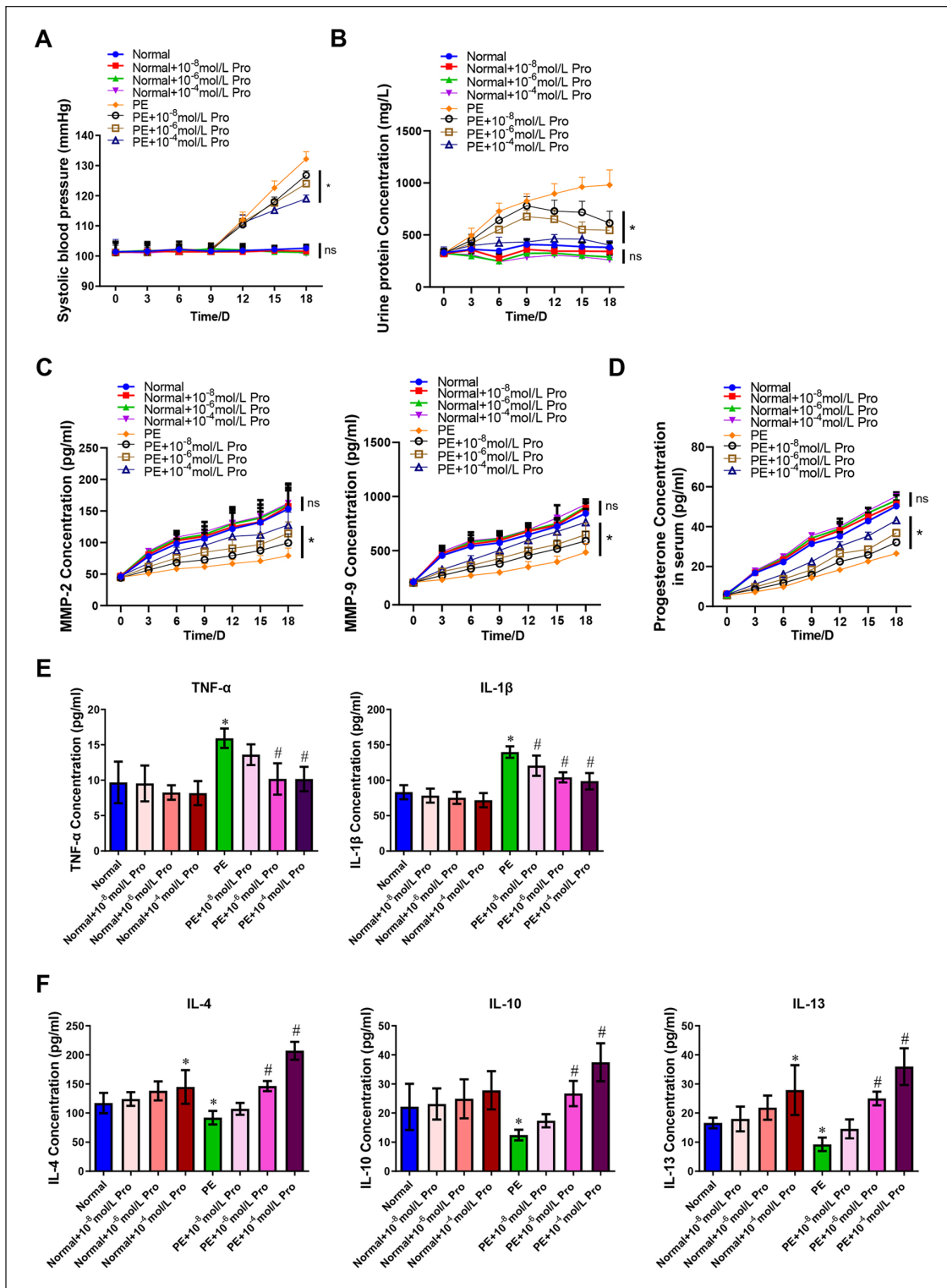
Figure 5. Effects of different concentrations of progesterone on blood pressure, urinary protein, cell invasion, progesterone levels and inflammatory factors in preeclampsia rats. In the Normal, Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone, preeclampsia, preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, preeclampsia+10^{-4}mol/L progesterone groups. (A) Blood pressure of rats from 0 to 20 days. (B) Urine protein of rats from 0 to 20 days. (C) MMP-2 and MMP-9 levels in serum from 0-20 days. (D) ELISA was used to detect the level of progesterone in serum from 0 to 20 days. (E) Pro-inflammatory factors TNF-α and IL-1β levels in serum were detected by ELISA. (F) Anti-inflammatory factors IL-4, IL-10 and IL-13 levels. ns: not significant; Pro: progesterone; PE: preeclampsia; ELISA: Enzyme-Linked Immunosorbent Assay; IL: interleukin; TNF: tumor necrosis factor; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9. *$P < 0.05$ vs Normal group; #$P < 0.05$ vs PE group.

progesterone in the preeclampsia group, cyclin D1, PCNA, MMP-2, and MMP-9 expression gradually increased with the progesterone concentration increased (Fig. 6B, C). Then, ELISA results showed that compared with Normal group, progesterone level in the preeclampsia group was significantly decreased. After the use of progesterone in the preeclampsia group, the level of progesterone gradually increased with the increase of progesterone concentration (Fig. 6D). The above results indicated that progesterone could promote cell proliferation and invasion, and its effect was more significant with the increase of progesterone concentration.

Different Concentrations of Progesterone Activated the PI3K/AKT Signaling Pathway and Inhibited the Inflammatory Response in Preeclampsia Rats

To explore whether different progesterone concentrations affect the PI3K/AKT signaling pathway, Western blot was used to detect AKT, p-AKT, PI3K, and p-PI3K expression in placenta tissues. Results showed that p-AKT and p-PI3K levels increased in the Normal$+10^{-8}$mol/L progesterone, Normal$+10^{-6}$mol/L progesterone, Normal$+10^{-4}$mol/L progesterone groups compared with the Normal group, while AKT and p-PI3K expression decreased in the preeclampsia group. After using progesterone in the preeclampsia group, AKT and p-PI3K expression gradually increased with the increase of progesterone concentration (Fig. 7A). Furthermore, Western blot was applied to detect the expression of pro-inflammatory factors TNF-α, IL-1β, and anti-inflammatory factors IL-4, IL-10, and IL-13 in placenta tissues. The results showed that compared with the Normal group, pro-inflammatory factors TNF-α and IL-1β levels decreased, anti-inflammatory factors IL-4, IL-10, and IL-13 levels increased in the Normal$+10^{-8}$mol/L progesterone, Normal$+10^{-6}$mol/L progesterone, Normal$+10^{-4}$mol/L progesterone groups. In the preeclampsia group, pro-inflammatory factors TNF-α and IL-1β levels increased significantly, and anti-inflammatory factors IL-4, IL-10, and IL-13 levels decreased significantly. After the use of progesterone in preeclampsia group, pro-inflammatory factors TNF-α and IL-1β levels gradually decreased, but anti-inflammatory factors IL-4, IL-10, and IL-13 levels gradually increased with the increase of progesterone concentration (Fig. 7B). The above results indicated that progesterone could improve inflammatory response, which involved the PI3K/AKT signaling pathway, and with the increase in progesterone concentration, its effect was more significant.

Discussion

Preeclampsia is a multifactorial disease characterized by impaired placental development, enhanced inflammation, and disordered placental steroid production[25]. However, the diagnosis and treatment of preeclampsia are limited. We investigated the effects of progesterone and the PI3K/AKT signaling pathway on HTR-8/Svneo cells by treating them with progesterone and PI3K inhibitor LY294002. The results showed that progesterone could enhance HTR-8/Svneo cell invasion ability by activating the PI3K/AKT signaling pathway and play a vital role in preeclampsia.

Similar to tumor cells, trophoblast cells exhibit invasive ability[26]. The important pathogenesis of early-onset preeclampsia is impaired invasion ability of trophoblast cells[27]. Abnormal infiltration of normal trophoblast cells can lead to abnormal development of uterine spiral arteries, which interferes with normal villi development and reduces placental perfusion. The traditional view is that progesterone improves endometrial receptivity by blocking the proliferative effect of estrogen, so as to facilitate the invasion of trophoblast cells, thereby enhancing the invasion of trophoblast cells[28]. Molecularly, progesterone plays a biological role mediated by the classical progesterone receptor A and nuclear progesterone receptor B. In recent years, the direct effect of progesterone on trophoblast cells has also been reported. Wilsher et al.[29] pointed out that progesterone could induce trophoblast cells to produce blocking antibodies, implement immune escape, and enhance trophoblast invasion in a cell-autonomous manner. Cui et al.[30] treated human embryonic trophoblast cells in vitro with progesterone and found that progesterone promoted the activation of c-Fos/c-Jun and improved the proliferation and adhesion of trophoblast cells. Nevertheless, the molecular mechanism is still unclear. In this study, we demonstrated that the use of progesterone promoted HTR-8/SVneo cells invasion. We found that progesterone supplementation decreased blood pressure and proteinuria. Here, our results showed that progesterone significantly attenuated the pathological changes in preeclampsia, in which progesterone could make the lumen of the uterine spiral artery larger in preeclampsia. Therefore, our findings indicated that progesterone supplementation might be an adjunct to treating preeclampsia by correcting the hormonal imbalance, which has important clinical significance.

PE is an excessive inflammatory reaction of women to pregnancy. Previous reports have indicated that impaired angiogenesis, increased oxidative stress, and hormonal and immunological changes in placental microenvironment might be the causes of placental defects in preeclampsia[25]. Impaired placental development will lead to persistent hypoxia at the fetus-mother interface and increased systemic inflammatory response[31]. Therefore, contrary to the mild systemic inflammation observed in normal pregnancy, severe preeclampsia was characterized by excessive systemic inflammation and activated innate immune response[32,33]. In the article of "assembly of cytoplasmic stress granules in placentas in women with preeclampsia," HTR-8/Svneo cells were treated with 10% serum from PE patients or normal pregnancy for 24 h to determine whether PE serum can induce the formation of stress granules[24]. Ma et al. found
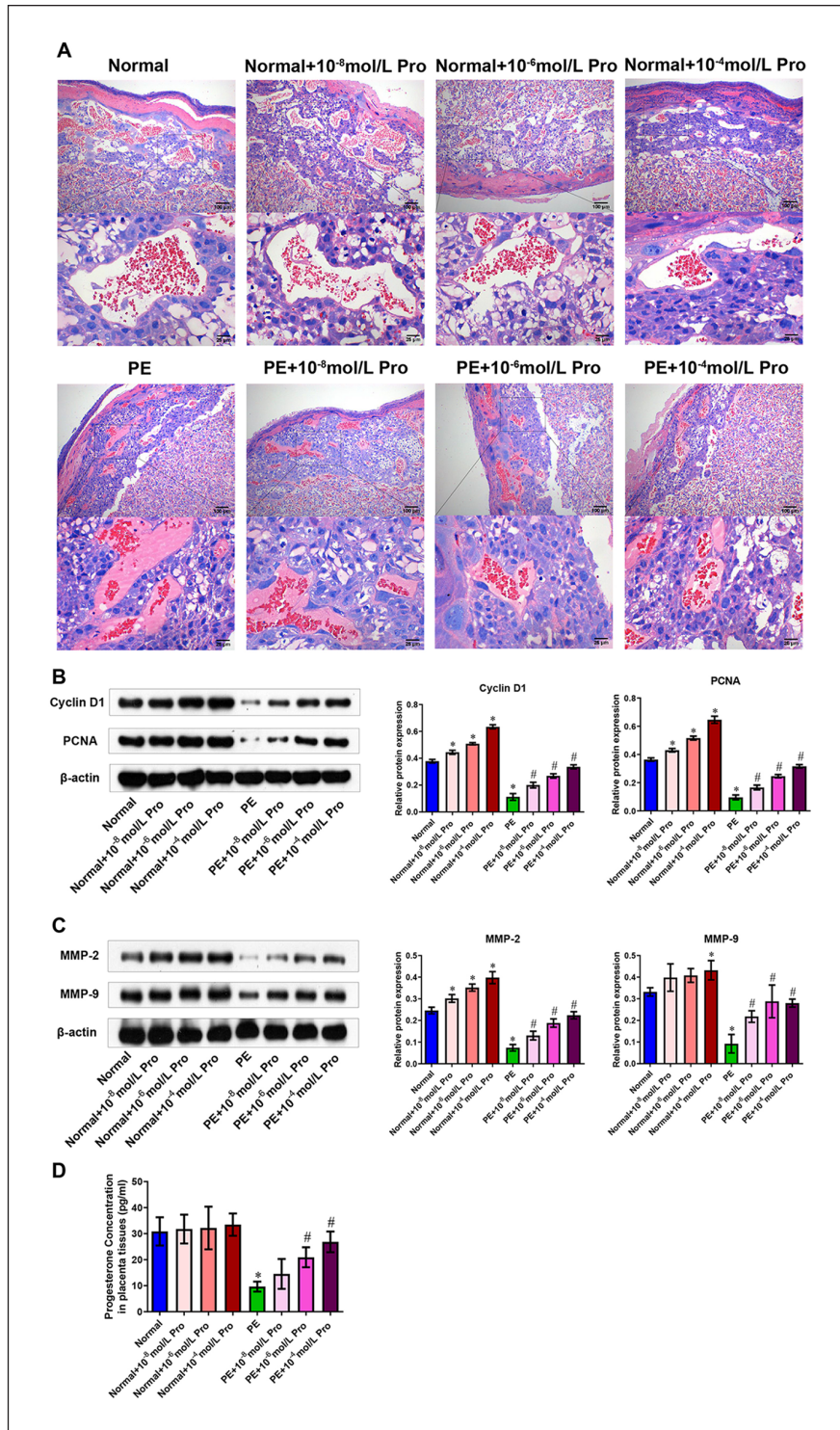
Figure 6. Different concentrations of progesterone promoted proliferation and invasion of placenta tissues in preeclampsia rats. In the Normal, Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone, preeclampsia, preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, preeclampsia+10^{-4}mol/L progesterone groups. (A) Morphological photograph of placenta tissues was stained with HE. (B) Cyclin D1 and PCNA expression in placenta tissues. (C) MMP-2 and MMP-9 expressions in placenta tissues. (D) Progesterone level in placenta tissues. Scale bar = 100 μm; The magnification was 100 times and 400 times respectively; Pro: progesterone; PE: preeclampsia; HE: Hematoxylin-Eosin; PCNA: proliferating cell nuclear antigen; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9. *$P < 0.05$ vs Normal group; #$P < 0.05$ vs preeclampsia group.
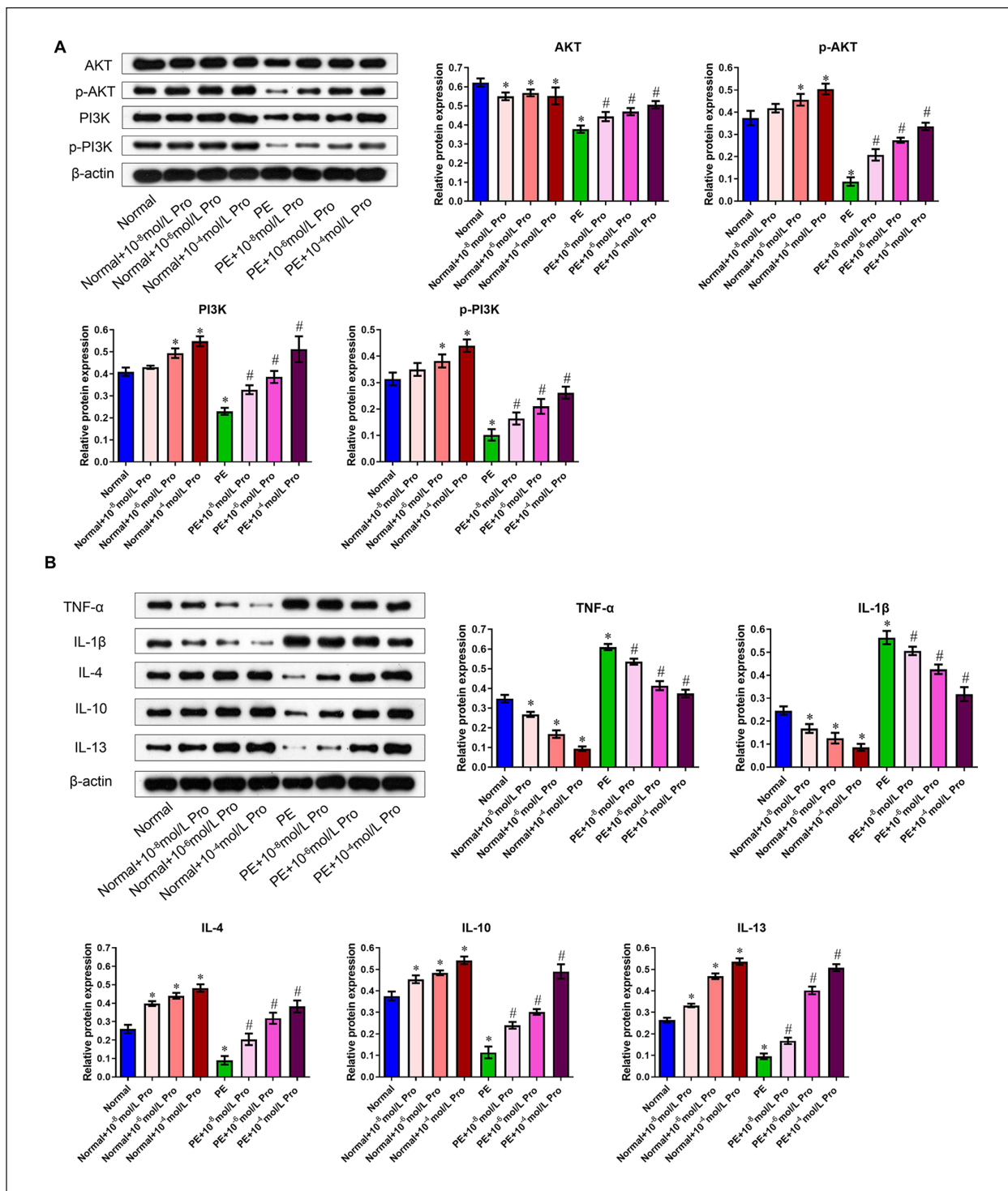
Figure 7. Different concentrations of progesterone activated the PI3K/AKT signaling pathway and inhibited the inflammatory response in preeclampsia rats. In the Normal, Normal+10^{-8}mol/L progesterone, Normal+10^{-6}mol/L progesterone, Normal+10^{-4}mol/L progesterone, preeclampsia, preeclampsia+10^{-8}mol/L progesterone, preeclampsia+10^{-6}mol/L progesterone, preeclampsia+10^{-4}mol/L progesterone groups. (A) AKT, p-AKT, PI3K, and p-PI3K expression in placenta tissues. (B) The expression of pro-inflammatory factors TNF-α and IL-1β and anti-inflammatory factors IL-4, IL-10, and IL-13 in the placenta tissues. Pro: progesterone; PE: preeclampsia; IL: interleukin; TNF: tumor necrosis factor; p-PI3K: phosphorylation of phosphoinositide 3-kinase; PI3K: phosphorylation of phosphoinositide 3-kinase; AKT: protein kinase B; p-AKT: phosphorylation of protein kinase B. *$P < 0.05$ vs Normal group; #$P < 0.05$ vs preeclampsia group.

serum from PE, not healthy pregnancy, can induce stress granules aggregation in HTR-8/Svneo cells, which can be explained by the higher levels of pro-inflammatory cytokines in PE serum. Our results showed that pro-inflammatory factors TNF-α and IL-1β levels decreased after using progesterone, but anti-inflammatory factors IL-4, IL-10, and IL-13 levels increased, which indicated that progesterone could improve the inflammatory response of preeclampsia.

Cyclin D1 was one of the main factors determining cell proliferation and apoptosis[34]. Cyclin D1 was expressed in trophoblast and extravillous trophoblast cells in the placenta tissues of normal pregnancy, suggesting that this protein might regulate the development and function of the human placenta[35]. In addition, the decrease of cyclin D1 in placenta tissues of preeclampsia indicated that the proliferation and migration of trophoblast cells might be reduced in preeclampsia[36]. PCNA was a nuclear protein synthesized in the G1/S phase of the cell cycle. It was associated with cell proliferation and played a vital role in DNA replication, cell division, and repair mechanisms[37]. Studies have shown that progesterone could up-regulate PCNA expression in the uterus of ovariectomized mice[38]. In this study, cyclin D1 and PCNA expression increased in normal pregnant and preeclampsia rats after using progesterone, indicating that progesterone could affect the cell cycle and promote trophoblast cell proliferation.

Matrix metalloproteinases (MMP) were critical modulators of vascular and uterine remodeling. The increase of MMP-2 and MMP-9 were related to vasodilation, placenta formation and uterine dilation during normal pregnancy[39]. Increased estrogen and progesterone production could increase MMP during pregnancy[20,40]. The decrease of MMP-2 and MMP-9 might cause decreased vasodilation, increased vasoconstriction, pregnancy-induced hypertension, and preeclampsia[41]. It was reported that reduced MMP-2 and MMP-9 interfere with the normal remodeling of uterine spiral arteries in early pregnancy, leading to the initial pathophysiological changes observed in preeclampsia[40]. We found that using progesterone after preeclampsia would increase MMP-2 and MMP-9 levels, but after using PI3K inhibitor LY294002, MMP-2, and MMP-9 levels decreased. It was consistent with trophoblast cell invasion changes. Therefore, the increase of MMP-2 and MMP-9 induced by progesterone is conducive to remodeling uterine spiral arteries.

The PI3K/AKT signaling pathway played an essential role in cell proliferation, migration, and invasion[42,43]. Previous studies showed that PI3K/AKT inhibitor LY294002 could eliminate bone marrow monocyte migration and invasion enhanced by adrenomedullin[44]. The literature has shown that human chorionic gonadotropin stimulated the migration and invasion of trophoblast cells via the PI3K/AKT signaling pathway involving its downstream effector MMP-2[17,20]. PI3K and AKT regulate trophoblast cell invasion by up-regulating MMP-9 and metalloproteinase-1 inhibitor expression

through epithelial growth factor[18]. Our Western blot results showed that after using progesterone in preeclampsia, proteins related to the PI3K/AKT signaling pathway expression increased, while that was decreased after using PI3K inhibitor LY294002. The above results showed that progesterone could activate the PI3K/AKT signaling pathway and enhance HTR-8/Svneo cell's invasion ability.

In conclusion, our results indicated that progesterone enhanced trophoblast cell invasion ability by activating the PI3K/AKT signaling pathway. In addition, progesterone reduced the progression of preeclampsia in pregnant rats in a concentration-dependent manner, which was related to the activation of the PI3K/AKT signaling pathway. Our study provided a theoretical basis for preeclampsia and helped to identify new treatment strategies for preeclampsia.

Author Contributions

Hongyu Liu and Yiling Ding designed the research, Ling Yu and Yiling Ding performed the research, Mei Peng and Yali Deng analyzed the data. All authors contributed to the writing and revisions. All authors reviewed the manuscript.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

Ethical approval to report this case series was obtained from "The Experimental Animal Ethics Review Committee of the Second Xiangya Hospital of Central South University (2020845)." Written informed consent was obtained from all participates.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the "The Ethics Review Committee of the Second Xiangya Hospital of Central South University (LYF2021065)" approved protocols.

Statement of Informed Consent

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

Declaration of Conflicting Interests

Funding

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Supplemental Material

Supplemental material for this article is available online.

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