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Activation of the ERK1/2 signaling pathway enhances proliferation and apoptosis of trophoblast in preeclampsia rats

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Abstract. – OBJECTIVE: The aim of this study was to investigate the influences of the extracellular signal-regulated kinase (ERK) 1/2 signaling pathway on preeclampsia rats as well as the proliferation and apoptosis of trophoblasts.

MATERIALS AND METHODS: A proper number of conceived rats were applied to prepare the preeclampsia model (group P). Meanwhile, others were enrolled as control group (group C). The differences in placental structure between the two groups were compared via hematoxylin-eosin (HE) staining. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were compared between the two groups as well. In addition, T25 cell lines were divided into three groups, including Control group, hypoxia/reoxygenation (H/R) group and H/R + Staurosporine group (an activator of the ERK1/2 signaling pathway). The protein expression of phosphorylated (p)-ERK1/2 in the aforementioned model groups and cells was detected through Western blotting. Cell apoptosis rate was determined by a flow cytometer. Moreover, methyl thiazolyl tetrazolium was utilized to measure the proliferative capacity of trophoblasts in the three groups. Transwell chamber assay was adopted to count the transmembrane cells.

RESULTS: The cells in group P were arranged disorderly. Group P had remarkably lower SOD activity but higher MDA content than group C ($p<0.05$). The protein expression levels of ERK1/2 and p-ERK1/2 in the placenta of group C were evidently higher than those of group P ($p<0.05$). Besides, the protein expression levels of ERK1/2 and p-ERK1/2 were markedly up-regulated in Control group when compared with H/R + Staurosporine group, with the lowest in H/R group ($p<0.05$). The proliferative capacity of cells was gradually enhanced in the three groups with the increase of culture time. Cell proliferation was the strongest in Control group, followed by H/R + Staurosporine group and H/R group ($p<0.05$). The apoptosis and death rates of cells were the highest in H/R group, followed by H/R + Staurosporine group and Control group ($p<0.05$). However, the number of transmembrane cells was the largest in Control group, followed by H/R + Staurosporine group and H/R group ($p<0.05$).

CONCLUSIONS: The ERK1/2 signaling pathway is associated with preeclampsia in rats, whose activation can enhance cell proliferation and weaken cell apoptosis.

Key Words:
ERK1/2 signaling pathway, Preeclampsia rats, Cell proliferation, Cell apoptosis.

Introduction

In recent years, all kinds of diseases become more and more ubiquitous despite constant advancement in modern medicine[1]. Preeclampsia refers to a disease with high incidence rate during pregnancy among women, which seriously poses a great threat to parturients[2,3]. Currently, much attention has been paid to the treatment and related theoretical studies of preeclampsia[4-7]. However, the specific pathogenesis of the disease has not been clearly identified. Li et al[6] has revealed that the occurrence of preeclampsia can strongly interfere in the growth and metabolism of embryonic trophoblasts. Combined treatment with drugs shows ideal efficacy in the initial stage of preeclampsia. However, it will cause various degrees of impacts and even the death of patients and fetuses if missing the best opportunity for treatment. With the development of modern molecular biology, the regulatory roles of signaling pathways in the pathological processes of multiple diseases have been gradually revealed[8]. In terms of the treatment of preeclampsia, Ahmed et al[9] has demonstrated that the HO-1 signaling pathway is highly correlated with many manifestations of preeclampsia patients through mod-

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ulating the activity of relevant enzymes, such as blood pressure change and proteinuria. It is also well known that preeclampsia, mainly originating from placental abnormalities in pregnant women, is triggered by multiple factors. Besides, differential genes leading to preeclampsia are implicated in processes such as cell proliferation, apoptosis and oxidative stress in patients[10]. However, the effects of the extracellular signal-regulated kinase (ERK) 1/2 signaling pathway on preeclampsia rats, the proliferation and apoptosis of placental trophoblasts, as well as the activity and content of oxidation indexes in serum are rarely reported.

In this research, therefore, placental tissues of preeclampsia rats and normal rats were observed and compared *via* hematoxylin-eosin (HE) staining. Oxidation indexes [superoxide dismutase (SOD) activity and malondialdehyde (MDA) content] in the serum of rats in the two groups were measured. Meanwhile, the protein expression levels of phosphorylated (p)-ERK1/2 in placental tissues of the two groups and three groups of T25 cell lines were determined and compared. In addition, the proliferative capacity of trophoblasts under three kinds of treatments was detected using methyl thiazolyl tetrazolium (MTT) assay. The apoptosis of T25 cell line in the three groups was examined, and the number of transmembrane cells was compared. Our findings aimed to systematically elaborate the influences of the ERK1/2 signaling pathway on preeclampsia rats and trophoblast proliferation and apoptosis.

Materials and Methods

Animals and Cell Lines

This investigation was approved by the Animal Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region Animal Center. Healthy female and male rats were purchased from Guizhou Weilan Jingling Biotech Co., Ltd. (Guiyang, China) and fed in People's Hospital of Xinjiang Uygur Autonomous Region in 2018. All the rats were fed with standard diets by special personnel to avoid the infection of other diseases. T25 trophoblast lines of rats were bought from Shanghai BinSuiBio Co., Ltd. (Shanghai, China) in 2016, and consumables of cell culture from FHDA (Ottawa, Canada).

Experimental Groups and Treatments

Experimental rats were placed in cages at a female/male ratio of 1:2, and pregnant rats were labeled by a certain mark. After labeling, conceived rats were grouped according to experimental requirements, with 15 rats in each group. 15 rats were injected with endotoxin using a small syringe (30 mg/kg per time) and set as preeclampsia group (group P). Meanwhile, the remaining rats were assigned into control group (group C) and injected with 0.85% normal saline instead of endotoxin. After that, a suitable size of embryonic tissues was cut, fixed in fixative and embedded. In addition, arterial blood samples were collected from both group C and group P.

Detection of Structural Changes In Placental Tissues Via HE Staining

Experimental samples were first taken out from liquid nitrogen. A part of the samples (0.5 cm thick) was gently placed in fixative (formalin), so as to prevent autolysis after cell death. 24 h later, the samples were rinsed in running water, and the rinsing time depended on the type of tissues. Next, the tissues were dehydrated in different concentrations of alcohol (2 h/gradient). During the process, paraffin blocks were boiled on an electric furnace for subsequent embedding. After replacement, embryonic tissues were embedded in paraffin and sliced to 6 μm-thick sections. Next, the sections were baked in a dryer (OTS, Wiesbaden, Germany) and spread. Structural changes in placental tissues of rats were finally observed and photographed under a light microscope (EVO MA 15/LS 15, Zeiss, Jena, Germany).

Determination of Oxidation Indexes In Rat Serum

The serum prepared as per method 1.2.1 was used to determine SOD activity and MDA content after centrifugation in a centrifuge in strict accordance with kit instructions. A spectrophotometer was used for reading.

Cell Line Grouping and Processing

T25 trophoblast cell lines were divided into three groups, including Control group, hypoxia/reoxygenation (H/R) group and H/R + Staurosporine group (Staurosporine is an activator of the ERK1/2 signaling pathway purchased from FHDA, Ottawa, Canada). The processing methods in H/R group and H/R + Staurosporine group were in line with those reported in literature[11]. During processing, the operating environment was controlled strictly to avoid pollution.

Detection of Protein Expression of p-ERK1/2 Via Western Blotting

ERK1/2 protein in each group was extracted according to the kit instructions. The concentration of extracted protein was determined by the Bradford method. Subsequently, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes by means of an electroporator (SDE102, Invitrogen, Carlsbad, CA, USA). After sealing for 30 min, the membranes were incubated with rat anti-p-ERK1/2 primary antibody at 4°C overnight. On the next day, the membranes were incubated with rabbit anti-rat horseradish peroxidase (HRP)-labeled secondary antibodies. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method. The results were finally photographed and analyzed.

Detection of Cell Proliferation Activity via MTT Assay

Experimental cells mentioned in method 1.2.4 were first cultured in culture plates for 24, 48 and 72 h. After that, a certain amount of MTT solution (6 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added in each, followed by incubation at 37°C under CO_2 and proper humidity. Cell culture medium was then poured. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was finally added and mixed evenly to dissolve the crystals, followed by reading *via* a micro-plate reader.

Measurement of Apoptosis Rate In Each Group

Experimental cells in the three groups were first cultured for a certain time period. After digestion with trypsin, the cells were harvested and washed with phosphate-buffered saline (PBS). Next, the cells were stained with Annexin V and Propidium Iodide (PI) according to the kit (FACSCalibur; BD Biosciences, Detroit, MI, USA) instructions. Cell apoptosis was finally examined.

Counting of Transmembrane Cells In Each Group

Transwell chambers were placed in a 96-well plate, and prepared gel was added into the chambers. A certain dose of cells in Control group, H/R group and H/R + Staurosporine group were added into the upper chamber. Meanwhile, cell culture medium containing 10% bovine serum was added to the lower chamber. After stable culture, HE staining was performed. Cells in each group were observed, photograph and counted. 10 non-overlapping fields of vision were randomly selected for each sample.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 15.0 (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis. Experimental data were presented as mean ± standard deviation ($\bar{x} \pm s$). Differences between two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

SOD Activity and MDA Content In Rat Blood

HE staining results manifested that the cells were arranged disorderly, the intercellular space was widened, and the basement membrane was thickened in group P when compared with group C. The detection of SOD activity and MDA content in rat blood revealed that SOD activity was remarkably weaker in group P than that in group C (14.32 U/mL *vs.* 27.43 U/mL, $p<0.05$) (Figure 1A). On the contrary, the content of blood MDA was significantly higher in group P than group C (2.17 nmol/mL *vs.* 1.46 nmol/mL, $p<0.05$) (Figure 1B).

Proteins Expression of ERK1/2 and p-ERK1/2 In Each Group

The protein expression levels of ERK1/2 and p-ERK1/2 in each group were shown in Figure 2A. The results indicated that the protein expression levels of ERK1/2 and p-ERK1/2 in the placenta of group C were evidently higher than those of group P (0.512 *vs.* 0.111, 0.797 *vs.* 0.131, $p<0.05$) (Figure 2B). Among the three groups of cells, the protein expression levels of ERK1/2 and p-ERK1/2 were markedly up-regulated in Control group when compared with H/R + Staurosporine group (0.531 *vs.* 0.419, 0.743 *vs.* 0.457, $p<0.05$), with the lowest in H/R group (0.127 and 0.146, respectively) ($p<0.05$) (Figure 3).

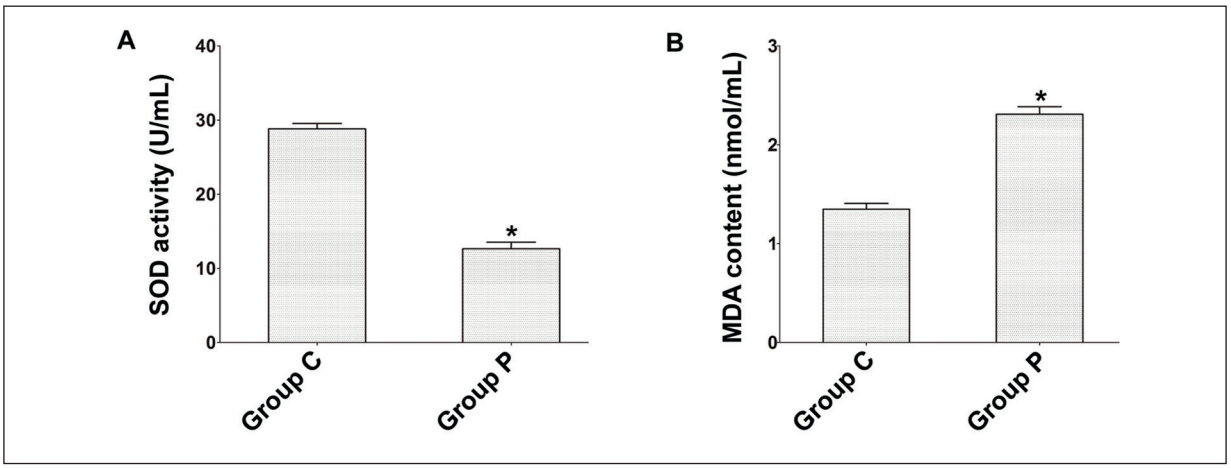

Figure 1. SOD activity and MDA content in rat blood. **A,** SOD activity in rat blood, **B,** MDA content in rat blood. *p<0.05: there is a significant difference compared with Control group.

Comparison of Proliferation Activity of Trophoblasts Among Three Treatment Groups

MTT assay was used to detect the proliferation activity of trophoblasts under three treat-

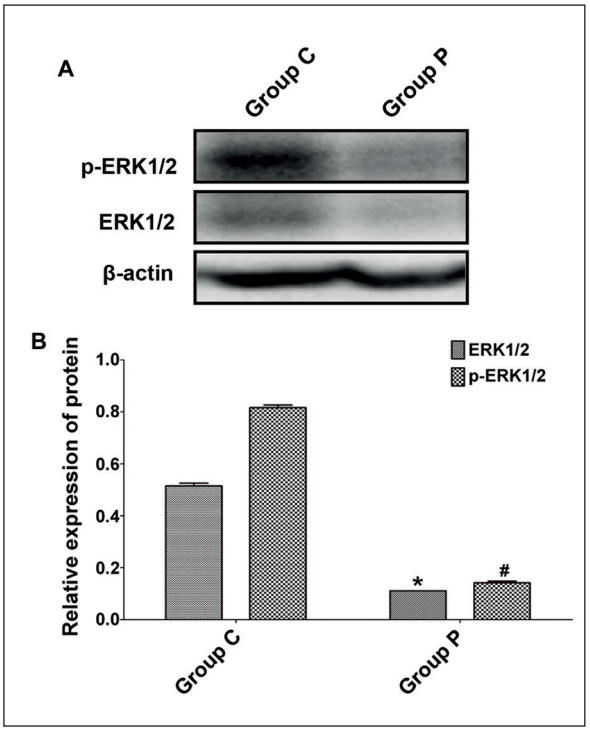

Figure 2. Protein expression levels of ERK1/2 and p-ERK1/2 in the two groups. **A,** Proteins expression levels of ERK1/2 and p-ERK1/2 detected via Western blotting. **B,** Quantitative comparisons of protein expression levels of ERK1/2 and p-ERK1/2 between the two groups. *p<0.05: there is a significant difference compared with Control group.

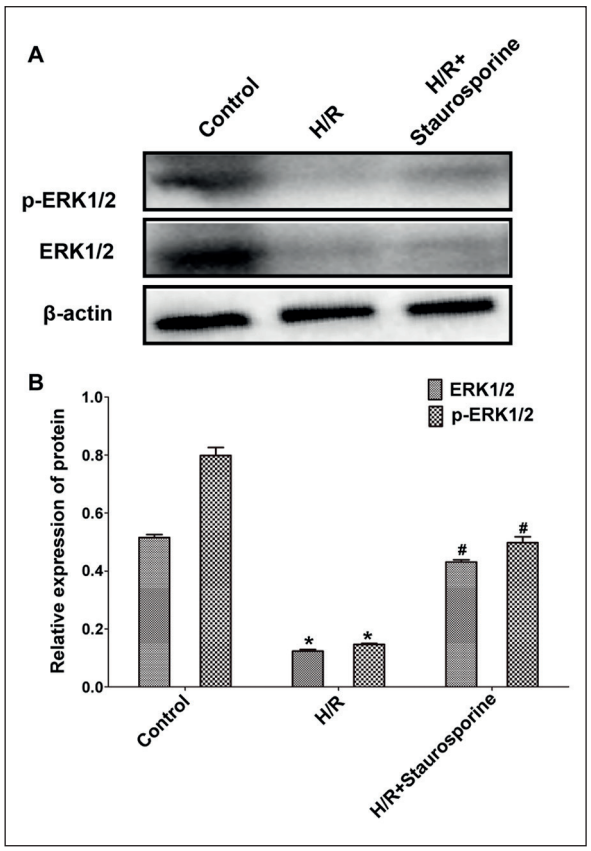

Figure 3. Protein expression levels of ERK1/2 and p-ERK1/2 in three groups of cells. **A,** Proteins expression levels of ERK1/2 and p-ERK1/2 detected via Western blotting. **B,** Quantitative comparisons of protein expression levels of ERK1/2 and p-ERK1/2 among the three groups. #p<0.05: there is a significant difference compared with Control group, and *p<0.05: the protein expression levels decline markedly in H/R group compared with those in H/R + Staurosporine group.

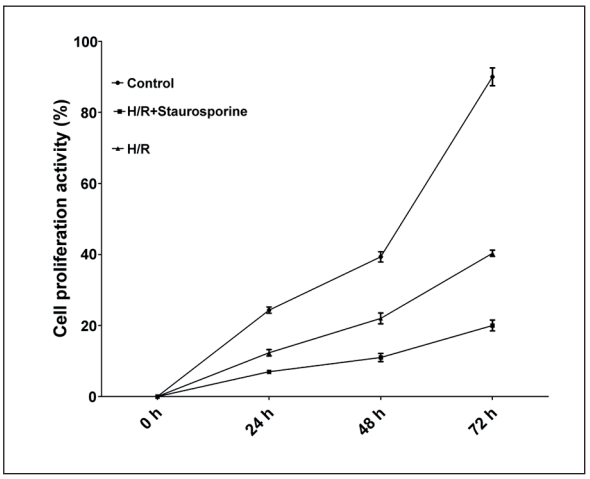

Figure 4. Proliferative capacity of trophoblasts in three treatment groups detected *via* MTT assay.

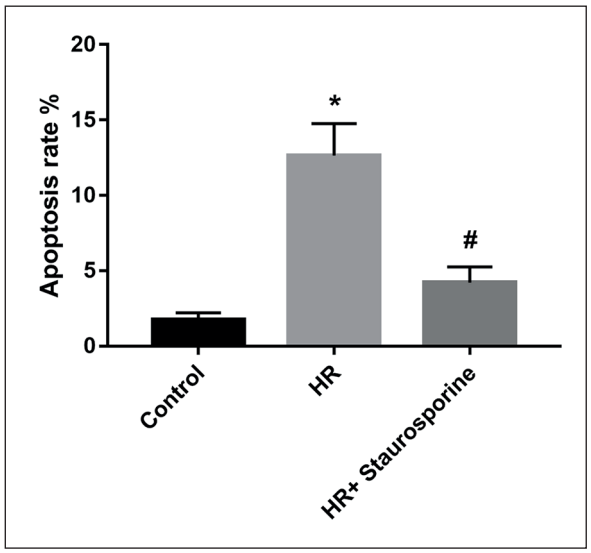

Figure 5. Trophoblast apoptosis in three treatment groups. **A,** Control group, **B,** H/R group, **C,** H/R + Staurosporine group.

ments in this research (Figure 4). The results showed that, during the whole culture period, the proliferation activity of cells was gradually enhanced with the increase of culture time in the three groups. Control group exhibited markedly stronger proliferation activity of trophoblasts than the other two groups (p<0.05). In addition, H/R + Staurosporine group exhibited notably stronger proliferation activity than H/R group (p<0.05).

Trophoblast Apoptosis In Three Treatment Groups

Subsequent results indicated that there were significant differences in the apoptosis rate of trophoblasts among the three treatment groups (Figure 5, Table I). In detail, the apoptosis rate of trophoblasts was the lowest in Control group (merely 4.6%), which was remarkably lower than that in H/R + Staurosporine group and H/R group (p<0.05). Moreover, the apoptosis rate in H/R + Staurosporine group was notably lower than that in H/R group (8.5% *vs.* 21.1%, p<0.05). In terms of cell death rate (Table I), it was also the lowest in Control group (1.2%), followed by H/R + Staurosporine group (3.7%) and H/R group (10.2%). In other words, Control group exerted significantly lower cell death rate than the remaining groups (p<0.05). Meanwhile, H/R + Staurosporine group displayed evidently decreased cell death rate when compared with H/R group (p<0.05).

Number of Transmembrane Trophoblasts In Three Treatment Groups

In this research, the number of transmembrane cells subjected to different treatments was recorded by means of transwell chamber experiment (Figure 6). The results demonstrated that the number of transmembrane cells was the largest in Control group (n=147.5), which was markedly larger than that in H/R + Staurosporine group (n=123.5) (p<0.05). However, the number of transmembrane cells was the smallest in H/R group (n=109.7), which was evidently smaller than the other two groups (p<0.05).

Table I. Statistics of cell apoptosis rate and death rate under different treatments.

Treatment group	Total cell number (n)	Percentage of apoptotic cells (%)	Percentage of dead cells (%)
Control group	10000	4.6c	1.2c
H/R group	10000	21.1a	10.2a
H/R + Staurosporine group	10000	8.5b	3.7b

a/b/c in the same column indicate significant differences between groups (p < 0.05).

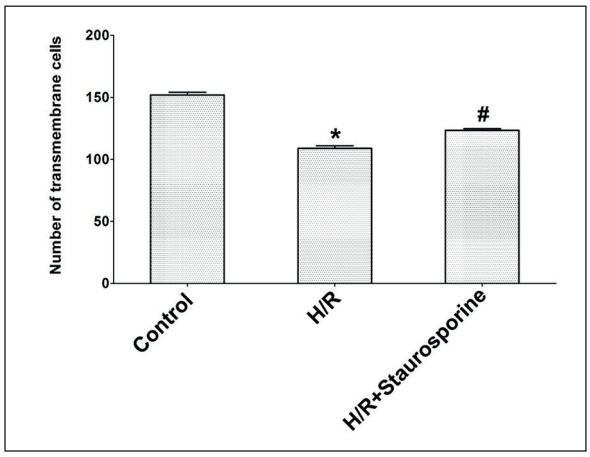

Figure 6. Number of transmembrane trophoblasts in different treatment groups $^{\#}p<0.05$: there is a significant difference compared with Control group, and $*p<0.05$: the number of transmembrane cells is reduced prominently in H/R group compared with that in H/R + Staurosporine group.

Discussion

Preeclampsia brings misfortune to numerous families over the past decades[12]. Multitudinous international researchers have paid much attention to the treatment of the disease[13]. In recent years, therapeutic drugs for preeclampsia, such as puerarin, have been discovered continuously. Additionally, it has been revealed that preeclampsia can induce many changes in the placenta of pregnant women. In this research, HE staining results manifested that disorderly arrangement of cells, increased intercellular space, and irregular thickening of basement membrane were observed in group P in contrast with those in group C. The activity of blood SOD decreased prominently. However, the content of MDA in the blood was markedly up-regulated in group P when compared with group C, which were consistent with the results of Procopciuc et al[14]. With the rapid development of molecular biology, studies on the regulation of gene expressions on preeclampsia rats and the metabolism of placental trophoblasts have been extensively carried out. Some scholars have found that activating or inhibiting specific signaling pathways in preeclampsia can effectively affect corresponding changes in these patients, including antioxidant substances in the blood, proteinuria, blood pressure and blood viscosity. In addition, alpha proteins can activate the ERK1 signaling pathway to induce the growth, development and differentiation of ventricular myocytes of rats. Such a process plays a crucial role in improving cardiomyocyte functions[15].

In this research, the results showed that the protein expression levels of ERK1/2 and p-ERK1/2 in the placenta of normal rats were remarkably elevated when compared with those of preeclampsia rats. Among the three groups of cells, the protein expression levels of ERK1/2 and p-ERK1/2 were notably higher in Control group than those in H/R + Staurosporine group and H/R group. On the whole, no significant difference was observed in the protein expression level of ERK1/2 in rat placenta between group C and Control group. Both groups exhibited prominently higher level than H/R + Staurosporine group. Meanwhile, no significant differences in the protein expression levels of ERK12 and p-ERK1/2 were observed between group P and H/R group, both of which were distinctly lower than those in the three aforementioned groups. All these results were consistent with the results of Lee et al[16]. Furthermore, studies have illustrated that the expressions of specified pathways have prominent influences on the proliferation of trophoblasts[17]. During the whole culture period, the proliferation activity of trophoblasts was significantly stronger in Control group, followed by H/R + Staurosporine group and H/R group. As for cell apoptosis rate and death rate, the apoptosis rate and death rate of trophoblasts were the lowest in Control group, followed by those in H/R + Staurosporine group and H/R group.

Conclusions

In summary, the novelty of this study was that the ERK1/2 signaling pathway is associated with preeclampsia in rats, whose activation can enhance cell proliferation and weaken cell apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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