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Pravastatin alleviates oxidative stress and decreases placental trophoblastic cell apoptosis through IL-6/STAT3 signaling pathway in preeclampsia rats

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Abstract. – OBJECTIVE: The aim of this study was to explore the effects of pravastatin on oxidative stress and placental trophoblastic cell apoptosis in preeclampsia rats via the interleukin (IL)-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway.

MATERIALS AND METHODS: Experimental rats were randomly assigned into three groups, including control group (C group), model group (M group) and pravastatin group (P group). The rat model of preeclampsia was successfully established. Blood pressure, urinary protein and nitric oxide (NO) as well as oxidative stress indicators in rats were detected at 7, 14 and 21 d, respectively. The content of serum IL-6 was determined via enzyme-linked immunosorbent assay (ELISA). The messenger ribonucleic acid (mRNA) expression of IL-6 in the placenta of rats in each group was detected using quantitative polymerase chain reaction (qPCR). Western blotting (WB) was used to determine the protein expression level of STATs in the placental tissues of rats. In addition, cell counting kit (CCK)-8 assay was conducted to detect the proliferation of rat placental trophoblasts.

RESULTS: The content of serum NO was (14.32±2.32) μM in M group, (28.37±3.32) μM in C group and (22.54±3.12) μM in P group, respectively. It was significantly elevated in P group compared with M group ($p<0.05$). Blood pressure in M group was evidently higher than that in C group at 14 and 21 d ($p<0.05$). However, P group exhibited distinctly lower blood pressure than M group ($p<0.05$). No statistically significant differences were observed in the urinary protein of rats among all the three groups at 7 d ($p>0.05$). At 14 and 21 d, the content of urinary protein in M group was considerably higher than that in C group ($p<0.05$). However, P group had distinctly lower urinary protein content than M group ($p<0.05$). Compared with C group, the content of malondialdehyde (MDA) and advanced oxidation protein products (AOPP) rose significantly in M group, whereas the content of superoxide dismutase (SOD) declined remarkably ($p<0.05$). In comparison with M group, P group exhibited declined MDA and AOPP content and increased SOD content, with statistically significant differences between the two groups ($p<0.05$). The expression level of serum IL-6 in rats in M group was markedly higher than that in C group ($p<0.05$). Meanwhile, the expression level of serum IL-6 evidently declined in P group compared with M group ($p<0.05$). Compared with C group, the protein expressions of phosphorylated STAT1 (*p*-STAT1) and p-STAT3 were considerably up-regulated in M group ($p<0.01$). However, they decreased prominently in P group in comparison with M group ($p<0.01$). C group exhibited a remarkably worse proliferation ability of rat placental trophoblasts than C group ($p<0.01$). In comparison with M group, the proliferation ability of rat placental trophoblasts was evidently enhanced in P group ($p<0.05$). Flow cytometry results indicated that the apoptosis of trophoblastic cells increased significantly in M group compared with that in C group ($p<0.01$). However, it significantly declined in P group in comparison with M group ($p<0.05$).

CONCLUSIONS: Pravastatin can repress the IL-6/STAT3 signaling pathway to alleviate oxidative stress, improve preeclampsia and decrease the apoptosis of placental trophoblastic cells in preeclampsia rats.

Key Words:
IL-6/STAT3 signaling pathway, Pravastatin, Preeclampsia, Oxidative stress, Trophoblastic cells.

Introduction

Cell apoptosis, also termed programmed cell death, is the cellular mechanism of cell loss and morphological changes, such as the development and maintenance of homeostasis in embryonic tissues[1]. Such a mechanism affects the differentiation and proliferation of trophoblasts, thereby

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playing a pivotal role in placental development[2]. The apoptosis level of villous cytotrophoblastic cells has been proven to rise in placental pathology, including early pregnancy loss and preeclampsia[3]. Currently, the etiology of preeclampsia remains unknown, but placental abnormalities seem to be critical factors[4]. The abnormality of placental development may be the mechanism by which apoptosis and angiogenesis are altered[5]. Cell apoptosis is mediated by two types of apoptosis markers, including: pro-apoptotic and anti-apoptotic markers[6]. The homeostasis between pro-apoptotic markers such as B-cell lymphoma 2 (Bcl-2) associated X protein (Bax), Caspase-8 and Caspase-3 and anti-apoptotic markers, like Bcl-2 has an important implication for the survival and proliferation of cells[7]. In the early stage of pregnancy, the placenta develops in hypoxia. Meanwhile, angiogenesis and vascularization occur to establish maternal-fetal blood vessels. Upon completion of vascularization, maternal blood begins to flow in the intervillous space, thereby increasing oxygen tension that will be offset by active antioxidants in the placenta. Any deficiency in the antioxidant defense mechanism will potentiate oxidative stress[8,9]. Compared with women with normal blood pressure, oxidative stress increases significantly in preeclampsia women. It not only reduces material and fetal angiogenesis[10-12], but also up-regulates pro-apoptotic markers in the placenta[13], as well as induces placental abnormalities and angiogenesis impairment[14]. In recent years, the association between endothelial cell apoptosis and angiogenesis has been elucidated[15]. According to a study hypothesis, oxidative stress alters angiogenesis and apoptosis, and inter-correlated pathophysiological events in preeclampsia. Meanwhile, massive inflammatory factors such as interleukin (IL)-6 are released in preeclampsia as well[16]. Placental oxidative stress may be one of the pathophysiological characteristics of preeclampsia[17]. Oxidative stress activates multiple signaling pathways to restore the balance in the body, but if failed, the mechanism of apoptosis may be activated[18].

Statins are normally able to treat cardiovascular disease, reduce cholesterol content, diminish inflammation, restrain oxidative stress, and regulate vascular endothelial function[19]. In the present study, therefore, preeclampsia rats were treated with pravastatin to verify whether pravastatin could inhibit the IL-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway to relieve oxidative stress, improve preeclampsia and decrease the apoptosis of placental trophoblastic cells.

Materials and Methods

Materials

Pravastatin and nitric oxide test kit were obtained from Cayman (Ann Arbor, MI, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were provided by Gibco (Rockville, MD, USA). Penicillin-streptomycin solution was obtained from Ambionic (Austin, TX, USA). Goat anti-rabbit and IgG primary antibodies and rabbit anti-rat horseradish peroxidase (HRP)-labeled secondary antibodies for Western blotting (WB) were bought from Abcam (Cambridge, MA, USA). Propidium Iodide (PI) dyes and Annexin were purchased from ABM (Richmond, BC, Canada). The enzyme-linked immunosorbent assay (ELISA) kit for IL-6 in rats was also provided by Abcam (Cambridge, MA, USA). TRIzol reagent was obtained from Life Technologies (Gaithersburg, MD, USA). Reverse transcription kit was purchased from AbmgoodChina, Inc. (Shanghai, China). SYBR Green Master Mix was provided by Bio-Rad (Hercules, CA, USA).

A total of 20 10-week-old female Sprague-Dawley (SD) rats weighing 220-250 g and 12 11-week-old male SD rats weighing 250 g were used as research subjects. This investigation was approved by the Animal Ethics Committee of Peking University Animal Center.

Establishment of Animal Preeclampsia Model and Grouping

Female and male SD rats started to be co-housed at 2:1 at 4 p.m. under the controlled temperature and light in a closed environment. Vaginal smear assay was performed in female rats at 8 a.m. the next morning. Female rats were observed using a microscope (×10 or ×20), and keratinized and enucleated epithelial cells and accumulated sperms with long and flexed tails suggested successful pregnancy. Subsequently, female rats were intraperitoneally injected with 13 mg of desoxycorticosterone acetate, and with 6.5 mg of desoxycorticosterone acetate on the 7th and 15th d, respectively. During pregnancy, the rats were fed with 0.9% normal saline. The animal model of preeclampsia was successfully established on the 18th d. All rats were divided into 3 groups, including: blank control group (C group, injected with 2 mL of normal saline daily), animal model group

(M group, injected with 2 mL of normal saline daily), and pravastatin group (P group, injected with 1 mg/d pravastatin daily).

For the following experiments, the rats were fed with the same diets in the same environment.

Detection of Blood Pressure and Serum NO In All Groups of Rats

Blood pressure of rats in the three groups was detected in the morning at 7, 14 and 21 d after pregnancy, respectively.

Serum samples of rats were first extracted. Optical density (OD) at the wavelength of 540 nm was detected by a micro-plate reader. The content of serum NO in each group of rats was finally calculated based on the OD value.

Detection of Urinary Protein In All Groups of Rats

24-h urine was first collected from rats in the three groups at 7, 14 and 21 d after pregnancy. The content of urinary total protein was determined by pyrogallol red colorimetry using an automatic biochemical analyzer.

Detection of Oxidative Stress Indicators In the Placental Tissues In All Groups of Rats

The content of malondialdehyde (MDA), advanced oxidation protein products (AOPP) and superoxide dismutase (SOD) in the placental tissues of rats in each group was determined by described methods in literature[20].

Sampling of Tissues and Blood In All Groups of Rats

The rats were anesthetized *via* intraperitoneal injection of 5% lidocaine hydrate at 9 a.m. on the 22nd day of pregnancy. Blood samples were drawn from the heart, followed by centrifugation. The supernatant was preserved at -70°C for use. Subsequently, the placenta was taken from rats in each group, and rat placental trophoblasts were extracted. Finally, the placenta was stored at -70°C for the following experiments.

Culture of Rat Placental Trophoblasts and Detection of Cell Proliferation Via Counting Kit (CCK)-8 Assay

Rat placental trophoblasts were cultured in complete DMEM containing 11.0% FBS and 1% penicillin-streptomycin in a thermostatic incubator with CO_2 at the volume percentage of 5%. When the confluence was 70-80%, the cells were sub-cultured. The above-mentioned rat placental trophoblasts collected from the three groups were separately cultured for 72 h. Afterwards, the cells were diluted to cell suspension at 1×10^6 cells/mL. The resulting cell suspension was incubated with 10% CCK-8 solution in a thermostatic 5% CO_2 incubator for 4 h in the dark. OD value of each well at 485 nm was determined by a micro-plate reader. The number of rat placental trophoblasts was calculated, and the growth curves of rat placental trophoblasts were finally plotted.

Determination of Serum IL-6 Content Via Enzyme-Linked Immunosorbent Assay (ELISA)

The level of serum IL-6 was determined according to the instructions of the ELISA kit.

Measurement of STAT Protein Expression Level in Placental Tissues of Rats by WB

STAT proteins were first extracted from placental tissues and washed with phosphate-buffered saline (PBS). After centrifugation for 5 min, the proteins were re-suspended in the buffer composed of 10 mmol/L HEPES/KOH, 2 mmol/L $MgCl_2$, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L KCl, and 1× protease inhibitor (pH=7.9), followed by centrifugation again for 30 s. The granular nucleus was washed with PBS for three times, gently re-suspended in ice-cold saline buffer containing 50 mmol/L HEPES/KOH, 50 mmol/L KCl, 300 mmol/L NaCl, 0.1 mmol/L EDTA, 10% glycerol, and 1× protease inhibitor (pH=7.9), ultrasonicated for 30 s and further centrifuged for 5 min. Protein expression levels of STAT were finally determined in strict accordance with WB.

Flow Cytometry Analysis of Cell Apoptosis and Cycle Distribution

Flow cytometry analysis was performed to evaluate the apoptosis of placental trophoblastic cells. Early preparations were made, and the assay was performed according to the instructions of the Annexin V-FITC apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry analysis was finally completed using FACScan system (Becton Dickinson, Mountain View, CA, USA) and CellQuest software (Becton Dickinson, San Jose, CA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 software (Chicago, IL, USA) was used for all

statistical analysis. Experimental data were presented as mean ± standard deviation ($\bar{\chi}$±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). $^{*}p<0.05$ and $^{**}p<0.01$ represented that the differences were statistically significant.

Results

Effects of Pravastatin on NO and Blood Pressure of Preeclampsia Rats

As shown in Figure 1, compared with C group [(28.37±3.32) μM], the content of serum NO substantially declined in group M [(14.32±2.32) μM] ($p<0.01$). However, P group had significantly higher content of serum NO [(22.54±3.12) μM] than M group ($p<0.05$). Therefore, the level of serum NO is upregulated in preeclampsia rats treated with pravastatin. However, no statistically significant differences were observed in the blood pressure of rats among all the groups on the 7th day of pregnancy. At 14 and 21 d after pregnancy, blood pressure in M group was evidently higher than that in C group ($p<0.05$). However, P group exhibited distinctly lower blood pressure than M group after treatment with pravastatin ($p<0.05$) (Table I). It can be inferred that pravastatin can lower the blood pressure of preeclampsia rats.

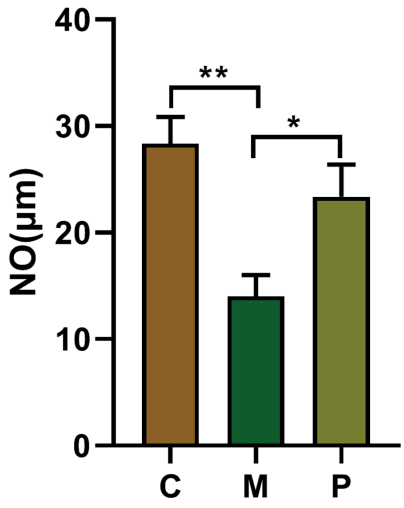

Figure 1. Content of serum NO in all groups of rats: Compared with that in C group, the content of serum NO substantially declined in M group ($p<0.01$). However, P group had significantly higher content of serum NO than M group ($p<0.05$).

Table I. Comparison of blood pressure of rats among all the groups ($\bar{\chi}$± s, mmHg).

Group	7 d	14 d	21 d
Age, yrs			
C	105.3±4.5	108.3±3.7	112.1±3.6
M	106.1±5.4	116.3±4.8^{x}	134.1±5.5^{x}
P	106.9±3.9	114.1±2.7^{xy}	121.1±4.1^{xy}

Note: $^{x}p<0.05$ *vs.* C group, and $^{y}p<0.05$ *vs.* M group.

Effect of Pravastatin on Urinary Protein Content In All Groups of Rats

As shown in Table II, no statistically significant differences were observed in the urinary protein of rats among all the groups at 7 d ($p>0.05$). At 14 and 21 d, the content of urinary protein in M group was evidently higher than that in C group ($p<0.05$). However, P group had distinctly lower urinary protein content than M group ($p<0.05$). The above findings suggest that pravastatin can decrease the discharge amount of urinary protein in preeclampsia rats.

Effects of Pravastatin on Oxidative Stress Indicators In the Placental Tissues of Preeclampsia Rats

Compared with C group, the content of MDA and AOPP significantly rose in M group, whereas the content of SOD substantially declined, showing statistically significant differences between the two groups ($p<0.05$). In comparison with M group, P group exhibited significantly decreased MDA and AOPP content and increased SOD content, with statistically significant differences between the two groups ($p<0.05$) (Table III). Therefore, pravastatin affects oxidative stress indicators in the placental tissues of preeclampsia rats.

Pravastatin Could Decrease the Expression of Serum IL-6 In Preeclampsia Rats

As shown in Figure 2, the expression level of serum IL-6 in M group was markedly higher than

Table II. Comparison of 24-h urinary protein content among all groups of rats ($\bar{\chi}$±s, mg).

Group	7 d	14 d	21 d
C	6.31±0.05	6.37±0.07	6.41±0.06
M	6.41±0.04	7.33±0.08^{x}	12.31±0.15^{x}
P	6.29±0.09	6.81±0.07^{xy}	8.15±0.11^{xy}

Note: $^{x}p<0.05$ *vs.* C group, and $^{y}p<0.05$ *vs.* M group.

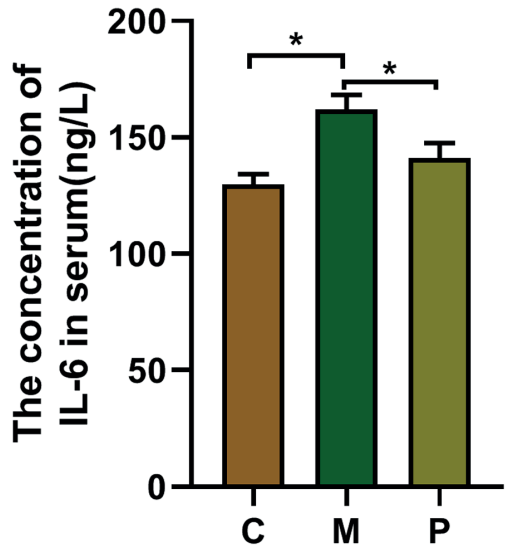

Figure 2. Expression of serum IL-6 in all groups of rats: The expression level of serum IL-6 in rats in M group was markedly higher than that in C group (p<0.05). However, the expression level of serum IL-6 evidently declined in P group compared with M group (p<0.05).

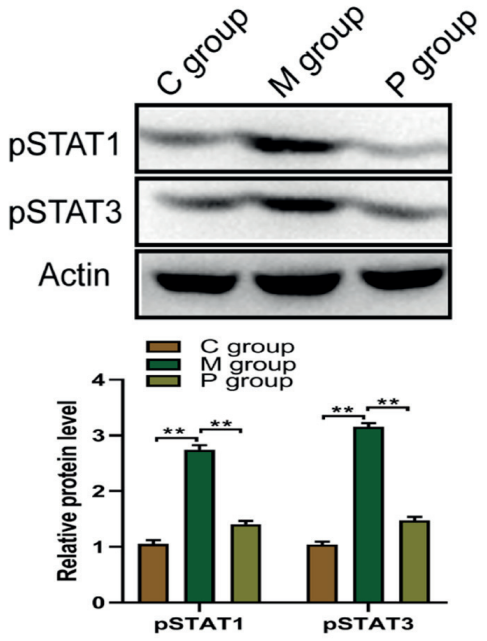

Figure 3. Effect of pravastatin on STAT protein expression in rats detected *via* WB: Compared with C group, the protein expressions of p-STAT1 and p-STAT3 were considerably raised in M group (p<0.01). However, they decreased prominently in P group in comparison with M group (p<0.01).

that in C group (p<0.05). However, the expression level of serum IL-6 evidently declined in P group compared with M group (p<0.05). These results suggest that pravastatin can decrease the expression of serum inflammatory factor IL-6 in preeclampsia rats.

Effect of Pravastatin on STAT Protein Expression In Rats Detected Via WB

Figure 3A and 3B presented the protein expression levels of phosphorylated STAT1 (p-STAT1) and p-STAT3 in each group of rats. Compared with C group, the protein expressions of p-STAT1 and p-STAT3 were considerably up-regulated in M group (p<0.01). However, they prominently decreased in P group in comparison with M group (p<0.01) (Figure 3A). Hence, pravastatin can substantially decrease the protein expressions of p-STAT1 and p-STAT3 in preeclampsia rats. In other words, it can inhibit the protein expression level of STAT.

Table III. Effects of pravastatin on oxidative stress indicators in the placental tissues of preeclampsia rats ($\bar{\chi}$±s, mg).

Group	MDA (mmol/ mg)	SOD (U/mg) (mmol/mg)	AOPP
C	5.11±0.06	162.37±7.17	5.23±0.04
M	13.41±0.14^{x}	87.33±4.35^{x}	11.43±0.24^{x}
P	7.89±0.06^{xy}	116.81±5.37^{xy}	9.02±0.11^{xy}

Note: ^{x}p<0.05 *vs.* C group, and ^{y}p<0.05 *vs.* M group.

Effects of Pravastatin on Trophoblastic Cells

According to the detection results (Figure 4), C group exhibited a remarkably worse proliferation ability of rat placental trophoblasts than C group (p<0.01). However, the proliferation ability of rat placental trophoblasts was evidently enhanced in P group compared with M group (p<0.05). Therefore, pravastatin can inhibit the apoptosis of trophoblastic cells and promote their proliferation in preeclampsia rats.

Pravastatin Inhibited Trophoblastic Cell Apoptosis

The changes in the apoptosis and cycle distribution of placental trophoblasts were shown in Figure 5. It was observed that the apoptosis of trophoblastic cells increased markedly in M group compared with C group (p<0.01). However, the apoptosis significantly declined in P group in comparison with M group (p<0.05). It can be inferred that pravastatin affects cell cycle to inhibit the apoptosis of trophoblastic cells.

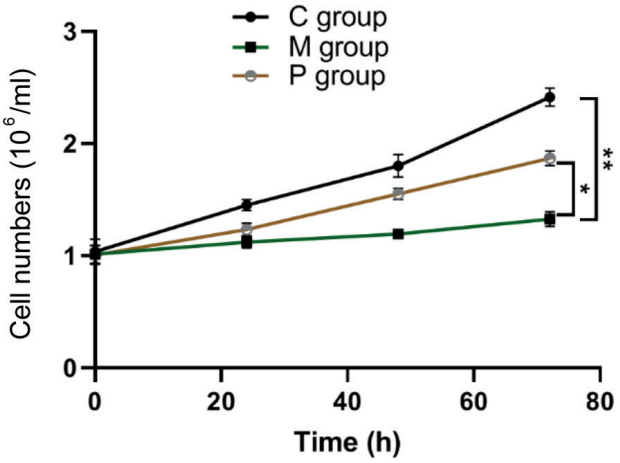

Figure 4. Effects of pravastatin on trophoblastic cells: M group exhibited a remarkably worse proliferation ability of rat placental trophoblasts than C group (p<0.01). In comparison with M group, the proliferation ability of rat placental trophoblasts was evidently enhanced in P group (p<0.05)..

Discussion

Preeclampsia, a common pregnancy disease, is characterized by such symptoms as small vessel spasm and reduced organ perfusion. It greatly endangers the life and health of pregnant women and fetuses. Over 50,000 people die of preeclampsia worldwide every year[21], the morbidity rate of which reaches up to 6% in China[1]. Currently, the etiology and pathogenesis of preeclampsia remain to be fully clarified. Preeclampsia has been classified into two phases in the medical field. It mainly exhibits pathophysiological changes in the first phase, with specific manifestations of abnormal trophoblast infiltration and reduced placental blood perfusion. However, it is mainly manifested as organ impairment, and specifically as various clinical phenomena in the second phase[22]. In recent years, it has been clinically believed that placental ischemia is the pathogenic basis of preeclampsia, thereby inducing the apoptosis of placental trophoblastic cells.

The effects of pravastatin on oxidative stress and placental trophoblastic cell apoptosis were explored in the present study. First, the content of serum NO in each group of rats was determined. It was found that the level of serum NO was significantly elevated in preeclampsia rats treated with pravastatin. Based on the results of blood pressure detection, pravastatin significantly decreased blood pressure of preeclampsia rats. Statins can not only treat cardiovascular diseases, but also reduce cholesterol content, alleviate inflammation, repress oxidative stress and modulate vascular endothelial function[19]. Subsequent results indicated that pravastatin decreased the discharge amount of urinary protein in preeclampsia rats. The content of oxidative stress indicators MDA, SOD and AOPP in placental tissues of rats was determined as well. It was discovered that the content

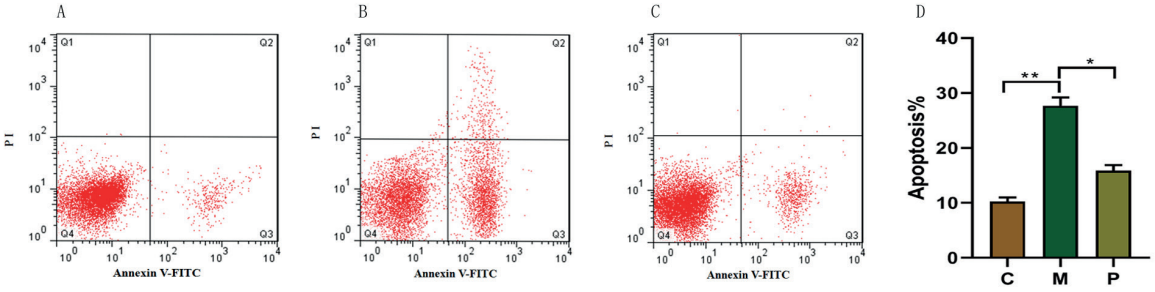

Figure 5. Effects of pravastatin on trophoblastic cell apoptosis and cycle distribution detected *via* flow cytometry: The apoptosis of trophoblastic cells increased significantly in M group compared with that in C group (p<0.01). However, it significantly declined in P group in comparison with M group (p<0.05).

of MDA and AOPP was significantly higher in M group than those in C group, whereas the content of SOD was substantially lower, showing statistically significant differences between the two groups ($p<0.05$). In comparison with M group, P group exhibited significantly declined MDA and AOPP content and increased SOD content, with statistically significant differences between the two groups ($p<0.05$). Therefore, pravastatin can increase the content of SOD and decrease that of MDA and AOPP in preeclampsia rats, thereby weakening the effect of oxidative stress on preeclampsia rats. Sharp et al[23] have suggested that hypoxia and oxidative stress can lead to placental cell apoptosis. Longtine et al[24] have demonstrated that hypertension increases the apoptosis rate of placental cells. In this study, the expression of serum inflammatory factor IL-6 in each group of rats was detected using ELISA. The results discovered that pravastatin decreased the expression of serum IL-6 in preeclampsia rats to alleviate the symptoms of preeclampsia. Kasture et al[16] have shown that, in the case of preeclampsia, oxidative stress can not only affect angiogenesis and apoptosis, but also induce the release of massive inflammatory factors such as IL-6. WB results showed that pravastatin substantially decreased the protein expressions of p-STAT1 and p-STAT3 in preeclampsia rats. In other words, it inhibited STAT protein expression, probably reducing placental trophoblastic cell apoptosis. Moreover, rat placental trophoblasts proliferation was detected *via* CCK-8 assay. The results revealed that pravastatin repressed the apoptosis and promoted the proliferation of rat placental trophoblasts. Finally, the apoptosis of placental trophoblastic cells was detected by flow cytometry. The results indicated that pravastatin restrained the apoptosis of placental trophoblastic cells in preeclampsia rats. In summary, pravastatin probably down-regulates the IL-6/STAT3 signaling pathway to reduce the release of inflammatory factors, STAT1 protein expression, oxidative stress level, urinary protein content and blood pressure, as well as elevate blood NO level.

Conclusions

The novelty of this study was that pravastatin can inhibit the IL-6/STAT3 signaling pathway to alleviate oxidative stress, improve preeclampsia and decrease the apoptosis of placental trophoblastic cells in preeclampsia rats.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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