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ORIGINAL PAPER

# Paeoniflorin Alleviates $H_2O_2$-Induced Oxidative Injury Through Down-Regulation of MicroRNA-135a in HT-22 Cells

**Ailing Zhai[1] · Zeng Zhang[1] · Xiangjuan Kong[1]**

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## Abstract

Paeoniflorin (PF) has been reported to possess neuroprotective influences on cognitive dysfunction illness. In current research, we attempted to probe into the protective influences of PF against $H_2O_2$-induced damage and the underlying regulating mechanisms on hippocampal HT-22 cells. HT-22 cells were pretreated with PF, and then induced by $H_2O_2$. Afterwards, the influences of PF pretreatment were examined using CCK-8 assay, apoptosis assay, western blot and ROS assay, respectively. In addition, the expression of microRNA-135a (miR-135a) was analyzed and altered by qRT-PCR and cell transfection, respectively. After overexpression of miR-135a, the effects of miR-135a mimic on cell functions were detected again. Moreover, influences of $H_2O_2$, PF and miR-135a overexpression on JAK2/STAT3 and ERK1/2 signal pathways were further investigated. Further experiments verified that PF pretreatment alleviated $H_2O_2$-induced oxidative stress through increasing cell viability, inhibiting cell apoptosis, reducing ROS generation and activating JAK2/STAT3 and ERK1/2 pathways. Besides, expression of miR-135a was declined by PF pretreatment. Whereas, miR-135a mimic abrogated the protective effects triggered by PF pretreatment. These results indicated that PF can alleviate $H_2O_2$-induced oxidative stress by down-regulation of miR-135a via activation of JAK2/STAT3 and ERK1/2 pathways.

**Keywords** Paeoniflorin · Alzheimer disease · Cell apoptosis · ROS · JAK2/STAT3 pathway · ERK1/2 pathways

## Introduction

Alzheimer disease (AD) is an neurodegenerative illness featured by cognitive dysfunction and progressive memory decline with personality and behavioral abnormalities [1]. The incidence of AD has been increasing with the population ages. Previous studies indicated that the chief pathological characteristic of AD are the amyloid deposits deposition around neurons, as well as neurofibrillary tangles of Tau proteins and neuronal apoptosis in nerve cells [2]. The medical nursing work makes an important contribution to the management of AD treatment. In addition to this, majority of studies have focused on pharmacological treatment of AD, including donepezil, galantamine and rivastigmine. However, there were still many dropouts and adverse events occurred during use of these drugs, therefore, there is still an urgent need to find new and effective medicines for stabilizing or slowing decline in cognition and function [3].

Paeoniflorin (PF) is the main effective constituent of *Radix Paeoniae alba*. Previous studies demonstrated that PF possessed varieties of biological activities, such as amelioration of inflammation in osteoarthritis [4] and colitis [5], recanalization of thrombosis [6], amelioration of atherosclerosis [7], as well as inhibition of tumorigenesis in myeloma [8], glioma [9], gastric cancer [10] and pancreatic cancer [11]. Besides, a previous study conducted by Hu et al. indicated that PF might exert a certain effect on the nervous system [12]. Gu et al. reported that PF exhibited neuroprotective effect on AD via influencing Bcl-2/Bax protein expression and inflammation in transgenic mice models of AD [13]. Moreover, Kapoor et al. demonstrated that PF exerted the neuroprotective effects against glutamate-induced neurotoxicity via the Bcl-2/Bax pathway in PC12 cells [14]. Furthermore, Li et al. and Wang et al. verified that PF attenuated $A\beta_{25-35}$-induced neurotoxicity via

✉ Ailing Zhai
ailing02al@163.com

Zeng Zhang
zhengxiang2545664@126.com

Xiangjuan Kong
jinjing51276773@126.com

[1] Department of Psychiatry, Jining Psychiatric Hospital, No. 1 Jidai Road, Jining 272051, Shandong, China

preventing mitochondrial dysfunction in PC12 cells and SH-SY5Y cells [15, 16]. However, the underlying mechanism by which PF performs its neuroprotective effects in HT-22 cells still remains unclear.

MicroRNAs (miRNAs) are widely considered to be the potential biomarkers of AD. Literature review showed that miR-135a expression was altered in blood, several cerebrospinal fluid (CSF) and the brain, consistently. Therefore, miR-135a has been recognized as one of the potential biomarkers for AD [17]. Recent studies have reported that miR-135a possibly regulated the expressions of crucial AD-related proteins APP and BACE-1, besides, miR-135a expression in the serum and CSF of AD patients was significantly decreased [18], which was inconsistent with the results of subsequent research [19]. Research in atherosclerosis demonstrated that overexpression of miR-135a could inhibit oxidative stress through decreasing reactive oxygen species (ROS) level [20]. However, whether PF works in cells through regulating miR-135a remains unknown.

This research intended to explore the protective influences of PF against $H_2O_2$-induced oxidative injury on HT-22 cells, and tried to probe into the potential molecular mechanism. The cell viability, apoptosis and their related factors in HT-22 cells and miR-135a mimic transfected HT-22 cells were detected after $H_2O_2$ and PF treatment. The possible mechanism was uncovered by determining the expressions of key factors involved in JAK2/STAT3 and ERK1/2 pathways. These observations may offer a new perspective for future AD treatment.

## Methods and Materials

### Cell Culture

Hippocampal HT-22 cells were gotten from the American Type Culture Collection (ATCC, Rockville, USA). The cells were sustained in Dulbecco's modified Eagle's medium (DMEM, BBI Life Sciences Corporation, Shanghai, China) as monolayers, complemented with 10% heat-inactivated fetal bovine serum (FBS, BBI Solution, Crumlin, UK). The cells were sustained in an incubator which comprising 5% $CO_2$ at 37 °C. In order to avoid cell characteristics changes along with the prolonged culture time, all experimental cells used were between passages 15 and 25. To maintain exponential growth, trypsin/EDTA treatment was conducted with each cell suspension when subculturing every 2 days.

### Cell Treatment

To induce oxidative injury, the cells were dealt with gradient concentrations of $H_2O_2$ (50, 100, 150, 200, 300 μM) for 3 h to determine the optimal concentration. The control group was dealt with fresh medium free of $H_2O_2$.

PF (purity > 99%) was purchased from the Aladdin (Shanghai, China). PF was firstly dissolved in DMEM medium to 2 mg/mL without FBS (BBI Solution, Crumlin, UK) supplement and then was diluted to 50, 100, 150 and 200 μg/mL. Subsequently, HT-22 cells were exposed to series concentrations for determining the appropriate concentration. Afterwards, HT-22 cells were pretreated with optimal concentration of PF for 24 h and then induced by $H_2O_2$ treatment for determining the influences of PF pretreatment.

### Hydrogen Peroxide Production

After PF pretreatment, the production of $H_2O_2$ in the culture supernatant of HT-22 cells was measured employing Amplex Red hydrogen peroxide detection kit (Thermo Fisher Scientific, Massachusetts, USA) following the product instructions. In brief, cells were firstly incubated with PF for 24 h, subsequently the supernatant was removed and Kreb's Ringers (HIMEDIA LABORATORIES, India) was supplied and co-incubated with cells for 4 h. Afterwards, the culture supernatant was collected and incubated with the Amplex Red horseradish peroxidase reagent at 37 °C for 4 h. Finally, the fluorescence intensity of excitation/emission 530/590 nm was detected utilizing a fluorospectro photometer (HITACHI, Tokyo, Japan).

### Cell Counting Kit-8 (CCK-8) Assay

The cell viability of HT-22 cells was assessed using CCK-8 (BBI Solution, Crumlin, UK) method. After stimulation, the reagent was added into the culture medium, and the mixture was maintained in an incubator comprising 5% $CO_2$ + 95% air at 37 °C for 1 h. The absorbance at 450 nm was detected utilizing a Microplate Reader (Bio-Rad, Hercules, CA).

### Apoptosis Assay

Cell apoptosis analysis was measured employing FITC-Annexin V/PI double-staining technique. Cells were firstly rinsed in phosphate buffered saline (PBS) and subsequently immobilized in 70% ethanol. Afterwards, the immobilized cells were rinsed twice in PBS and stained with PI/FITC-Annexin V with the existence of 50 μg/ml RNase A (Thermo Scientific, Massachusetts, USA), and afterwards maintained at 25 °C in dark situation for 1 h. The proportion of apoptotic cells was determined by flow cytometry and then calculated utilizing FlowJo software.

### Western Blot

The proteins were taken out using RIPA lysis buffer (Sangon Biotech, Shanghai, China) in the presence of protease inhibitors (Sangon Biotech, Shanghai, China). The concentrations of proteins extracted were detected utilizing the BCA™ Protein Assay Kit (Thermo Scientific, Massachusetts, USA). The target proteins extracted were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently were transferred to polyvinylidene fluoride (PVDF) membranes (Solarbio, Beijing, China) for immunoblotting. After blocking in 5% bull serum albumin (BSA; Millipore) at 25 °C for 1 h, the spots were maintained overnight at 4 °C with primary antibodies directed against CyclinD1 (ab16663, abcam), p53 (ab131442, abcam), Cleaved-caspase 3 (ab49822, abcam), Cleaved-PARP (ab32064, abcam), t-JAK2 (ab39636, abcam), p-JAK2 (ab32101, abcam), t-STAT3 (ab137803, abcam), p-STAT3 (ab76315, abcam), t-ERK1/2 (ab17942, abcam), p-ERK1/2 (#4370, Cell Signaling Technology) and β-actin (ab8227, abcam). After rinsing with Tris-Buffered Saline Tween (TBST) 20 buffer, the PVDF membranes were maintained with secondary antibody (ab6721, abcam) labeled by horseradish peroxidase (HRP) at 25 °C for 1 h. After washing, the PVDF membrane was removed into the Bio-Rad ChemiDoc™ XRS system. Subsequently, Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added encircling the membrane. The signals were detected and analyzed utilizing Image Lab™ Software (Bio-Rad, Shanghai, China).

### ROS Assay

The cells were maintained with serum-free culture medium comprising 10 μM 2,7-dichlorofluorescein diacetate (DCFH-DA, aladdin, Shanghai, China) at 37 °C for 20 min in dark condition, then were plated into a 6-well plate. Subsequently, the cells were rinsed with PBS and digested with trypsin (Sangon Biotech, Shanghai, China) for sample collection. Then all samples were centrifuged and the supernatants were discarded. Precipitate was suspended in 500 μl PBS and fluorescent intensities were detected utilizing a flow cytometer (Beckman Coulter, USA).

### Cell Transfection

HT-22 cells were respectively transfected with miR-135a mimic and negative control (NC) mimic, synthesized by Sangon Biotech (Shanghai, China), utilizing Lipofectamine 3000 reagent (Invitrogen) according to the product instructions.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs were taken out from cells utilizing TRIzol reagent (Invitrogen) with the presence of DNaseI (Promega). The concentrations of the extracted RNAs were quantified with a Nanodrop 2000 system (Thermo Scientific, Massachusetts, USA). The cDNA was synthesized utilizing the MultiscribeRT kit (Applied Biosystems) and oligo (dT) primers. The reverse transcription conditions were 25 °C for 10 min, 48 °C for 30 min, and a final step 95 °C for 5 min. The relative expression of miR-135a was normalized to U6.

### Statistical Analysis

The results of these experiments, each repeated for three times, were presented as the mean ± standard deviation (SD). Statistical analyses were completed utilizing SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA). The *P*-values between two groups and muti-groups were respectively calculated using *LSD-t* test and one-way analysis of variance (ANOVA). $P < 0.05$ was considered as statistically significant.

## Results

### $H_2O_2$ Exposure Induced Oxidative Damage in HT-22 Cells

To determine the adequate $H_2O_2$ concentration for subsequent experiments, cell viability analysis was conducted following a 0–300 μM $H_2O_2$ treatment in HT-22 cells. CCK-8 assay results suggested that cell viability was significantly decreased at 100, 150, 200 and 300 μM ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) $H_2O_2$ treatment and exhibited as a concentration-dependent decrease. Because the cell viability was the closest to the value of IC50 when treated with 200 μM $H_2O_2$ (Fig. 1a), thus we chose this concentration to conduct the following experiments. The expressions of proliferation-related CyclinD1 and p53 were also examined, and the results demonstrated that $H_2O_2$ treatment significantly decreased CyclinD1 expression ($P < 0.05$) whereas notably increased p53 expression ($P < 0.01$) compared with control (Fig. 1b, c). In Fig. 1d, the percentage of apoptotic cells in $H_2O_2$-treatment group was significantly higher than control ($P < 0.001$). Besides, the expressions of apoptosis-related proteins Cleaved-PARP and Cleaved-Caspase-3 was detected. The results revealed that both the expressions of Cleaved-Caspase-3 ($P < 0.001$) and Cleaved-PARP ($P < 0.01$) were markedly up-regulated by $H_2O_2$ treatment compared to control (Fig. 1e, f). Meanwhile, $H_2O_2$ treatment resulted in significant increase of ROS generation

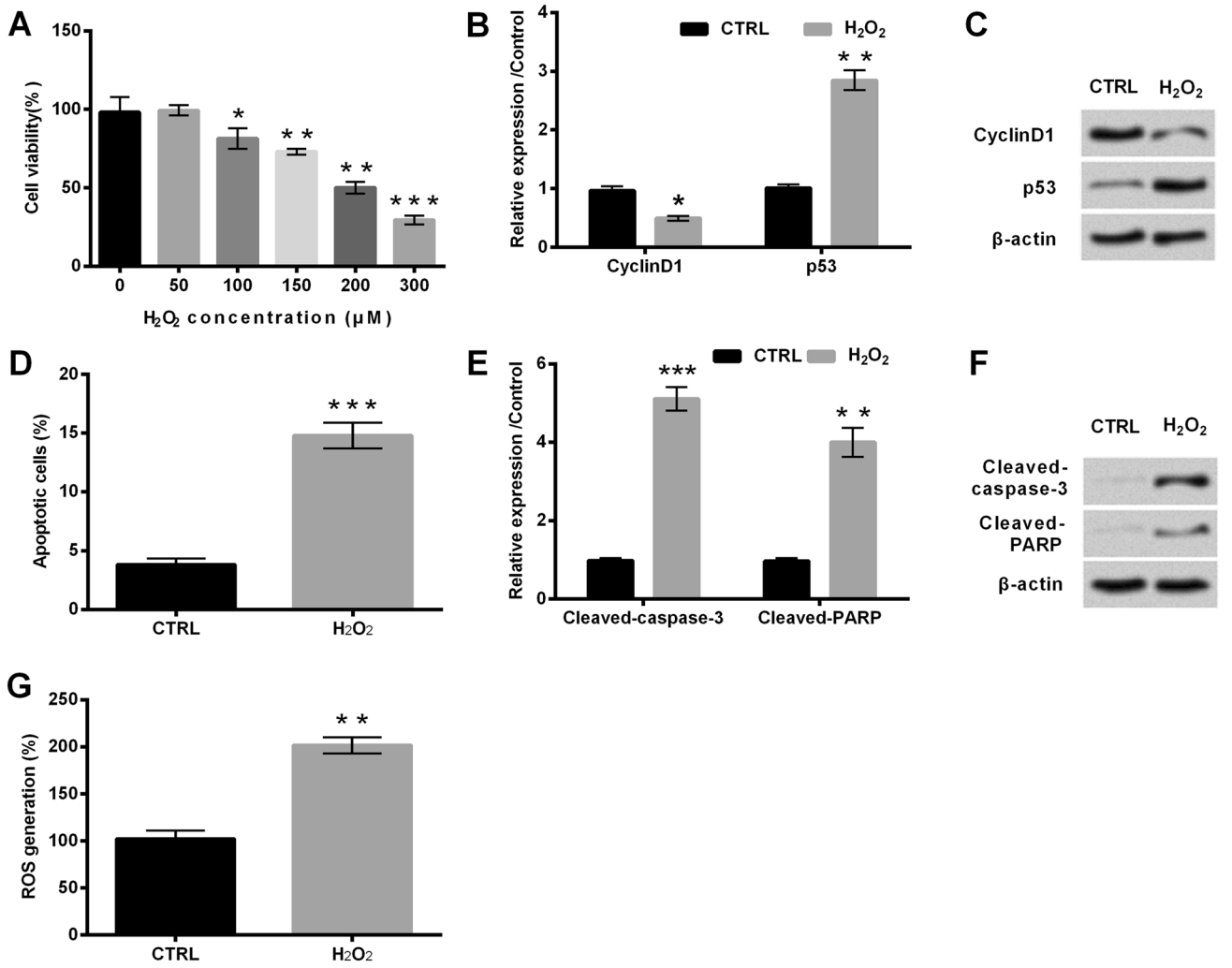


**Fig. 1** $H_2O_2$ treatment induced HT-22 cells oxidative injury. **a** $H_2O_2$ treatment (100, 150, 200 and 300 μM) notably decreased cell viability and the results showed in a concentration-dependent manner. **b**, **c** $H_2O_2$ treatment remarkably decreased the expression of apoptosis-related CyclinD1 and markedly enhanced the expression of p53. D. $H_2O_2$ treatment dramatically increased the proportion of apoptotic cells. **e**, **f** $H_2O_2$ treatment dramatically enhanced the expressions of apoptosis-related Cleaved-caspase-3 and Cleaved-PARP. **g** $H_2O_2$ treatment markedly increased the generation of ROS. *CTRL* control, *PARP* poly-ADP-ribose polymerase, *ROS* reactive oxygen species. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

compared to non-treated group ($P<0.01$, Fig. 1g). These data suggested that the injury model of AD was preliminarily constructed in HT-22 cells.

## PF Pretreatment Alleviated $H_2O_2$-Induced Oxidative Damage in HT-22 Cells

HT-22 cells were dealt with gradient concentrations of PF (0, 50, 100, 150 and 200 μg/mL) and then CCK-8 assay was carried out to assess the influences of PF treatment. The results illustrated that PF treatment had no significant influence on cell viability (Fig. 2a). Then, the influences of PF on $H_2O_2$-stimulated HT-22 cells were studied. $H_2O_2$-induced HT-22 cells were pre-treated with series of concentrations of PF (0, 50, 100 and 200 μg/mL). As shown in Fig. 2b, cell viability was notably increased by PF pretreatment compared with $H_2O_2$-treated group and exhibited in a concentration-dependent way ($P<0.05$ or $P<0.01$). Because the most effective concentration was 200 μg/mL, hence, we chose this concentration to conduct subsequent experiments. Detection of expressions of proliferation-related proteins showed that PF treatment itself prominently promoted the expression of CyclinD1 ($P<0.05$), while notably repressed the expression of p53 ($P<0.01$). Meanwhile, further results illustrated that PF pretreatment alleviated $H_2O_2$-triggered suppression effect on CyclinD1 expression ($P<0.05$) and the promotion effect on p53 expression ($P<0.05$, Fig. 2c, d). Besides, PF individually treatment had no significant influence on cell apoptosis,

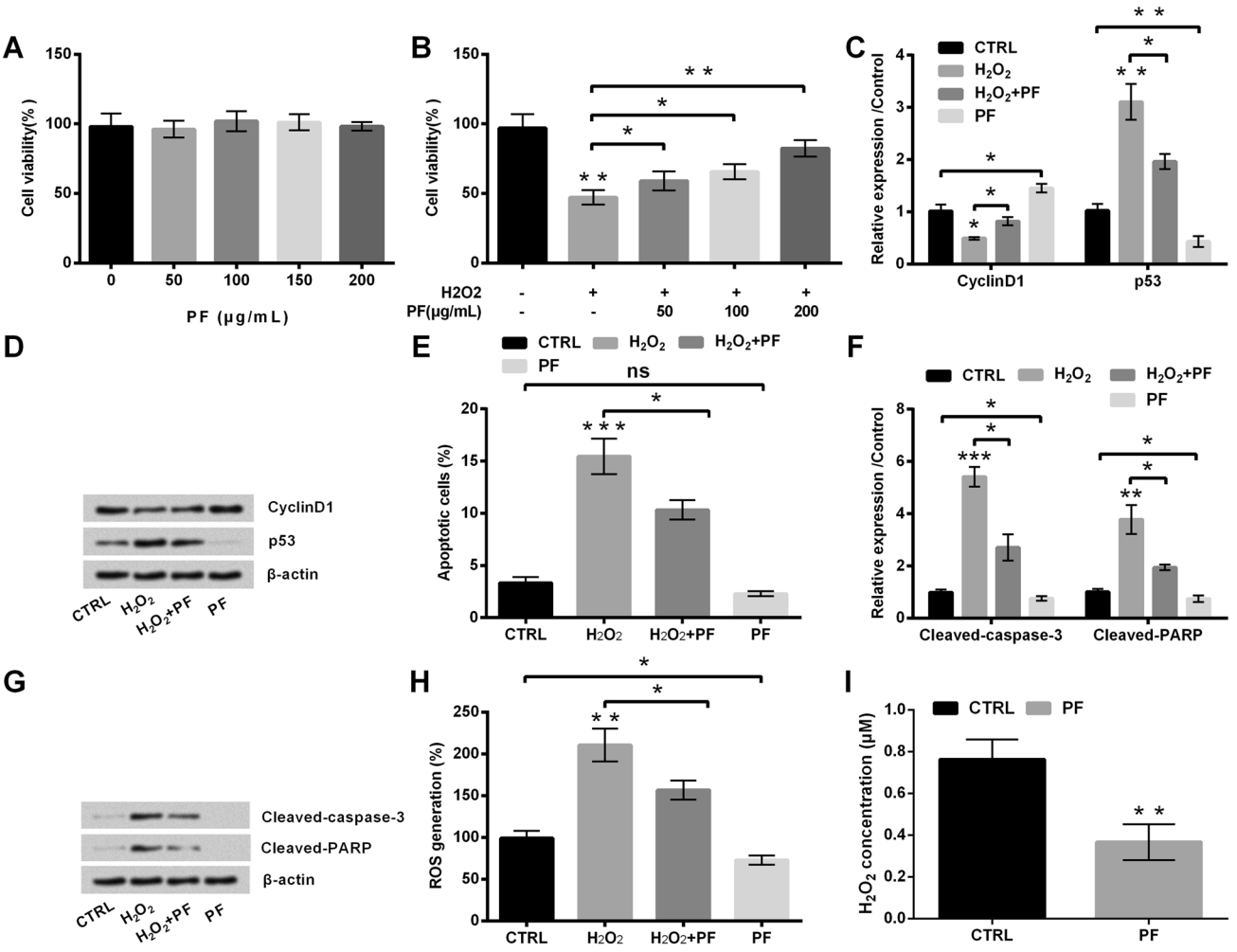


**Fig. 2** PF pretreatment alleviated $H_2O_2$-induced oxidative injury in HT-22 cells. **a** PF treatment (0, 50, 100, 150 and 200 μg/mL) had no significant influence on cell viability. **b** PF pretreatment remarkably alleviated $H_2O_2$-induced inhibitory effect on cell viability and exhibited in a dose-dependent way. **c**, **d** PF treatment notably increased the expression of apoptosis-related CyclinD1 and decreased the expression of p53. Besides, PF pretreatment markedly relieved $H_2O_2$-induced inhibitory effect on CyclinD1 expression and the promotion effect on p53 expression. **e** PF treatment had no notable influence on cell apoptosis. While PF pretreatment remarkably alleviated $H_2O_2$-induced promotion effect on cell apoptosis. **f**, **g** PF treatment notably decreased the expressions of apoptosis-related Cleaved-caspase-3 and Cleaved-PARP. PF pretreatment markedly relieved $H_2O_2$-induced promotion effects on the expressions of Cleaved-caspase-3 and Cleaved-PARP. **h** PF treatment significantly reduced the generation of ROS. PF pretreatment observably relieved the promotion effect on ROS generation. **i** PF treatment observably reduced the production of $H_2O_2$. *CTRL* control, *ns* no significance, *PF* Paeoniflorin, *PARP* poly-ADP-ribose polymerase, *ROS* reactive oxygen species.*$P<0.05$; **$P<0.01$; ***$P<0.001$

while notably reduced the expressions of apoptosis-related Cleaved-caspase-3 and Cleaved-PARP compared to control group (both $P<0.05$). Additionally, PF pretreatment dramatically relieved the promotion effects on cell apoptosis ($P<0.05$, Fig. 2e), and remarkably eliminated the enhanced expressions of Cleaved-caspase-3 and Cleaved-PARP compared to $H_2O_2$-treated group (both $P<0.05$, Fig. 2f, g). For ROS assay, we observed that the $H_2O_2$-triggered increased effect on ROS generation was significantly attenuated by PF pretreatment ($P<0.05$). In addition, PF exposure itself significantly decreased ROS production ($P<0.05$, Fig. 2h) and $H_2O_2$ generation ($P<0.01$, Fig. 2i). In brief, these results led to a conclusion that PF possessed the cytoprotection and ROS attenuation effects on HT-22 cells.

## PF Pretreatment Promoted Activation of JAK2/STAT3 and ERK1/2 Pathways

To uncover the potential regulation mechanism, we investigated some of the key factors involved in the JAK2/STAT3 and ERK1/2 pathways. Results displayed that the expressions of p/t-JAK2 and p/t-STAT3 were notably enhanced by PF exposure (both $P<0.01$), while were markedly inhibited by $H_2O_2$ treatment (both $P<0.05$). However, the

inhibitory effects were remarkably reversed by PF pretreatment ($P<0.05$, Fig. 3a, b). The same expression pattern also appeared in the ERK1/2 signaling pathway exhibiting as PF exposure notably elevated the expression of p/t-ERK1/2 ($P<0.05$), while $H_2O_2$ treatment observably suppressed p/t-ERK1/2 expression ($P<0.01$), whereas, this suppression effect was markedly reversed by PF pretreatment ($P<0.05$, Fig. 3c, d). Therefore, these results pointed out that PF pretreatment could promote the activation of the JAK2/STAT3 and ERK1/2 pathways.

## PF Pretreatment Promoted Down-Regulation of miR-135a

To clarify the function of miR-135a in AD, we tested the expression of miR-135a in $H_2O_2$, PF and $H_2O_2$ + PF treated HT-22 cells, respectively. The results turned out to be that miR-135a expression was remarkably inhibited by PF exposure compared to control ($P<0.05$), while was markedly enhanced in the $H_2O_2$-treated group compared to control ($P<0.01$). However in the $H_2O_2$ + PF treated group, the promotion effect on miR-135a expression induced by $H_2O_2$ exposure was markedly alleviated by PF pretreatment ($P<0.05$, Fig. 4). These results implied that PF exposure had significant influences on miR-135a expression.

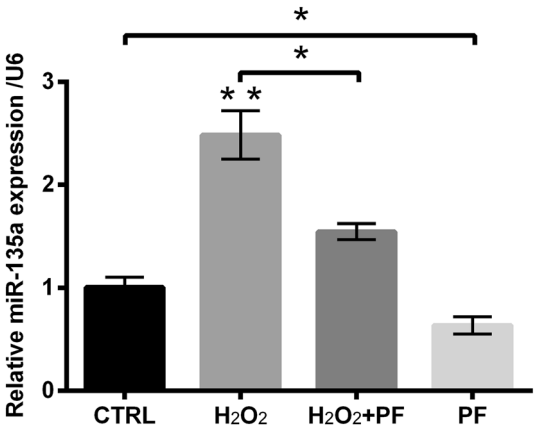


**Fig. 4** PF pretreatment down-regulated the expression of miR-135a in $H_2O_2$-induced HT-22 cells. PF treatment notably decreased the expression of miR-135a. Besides, $H_2O_2$ treatment dramatically enhanced the miR-135a expression, while PF pretreatment observably relieved the promotion effect. *CTRL* control, *PF* Paeoniflorin, *miR-135a* microRNA-135a. $^{**}P<0.05$; $^{***}P<0.01$

## PF Pretreatment Attenuated $H_2O_2$-Triggered Oxidative Injury in HT-22 Cells Through Down-Regulation of miR-135a

For further investigation of the function of miR-135a in the

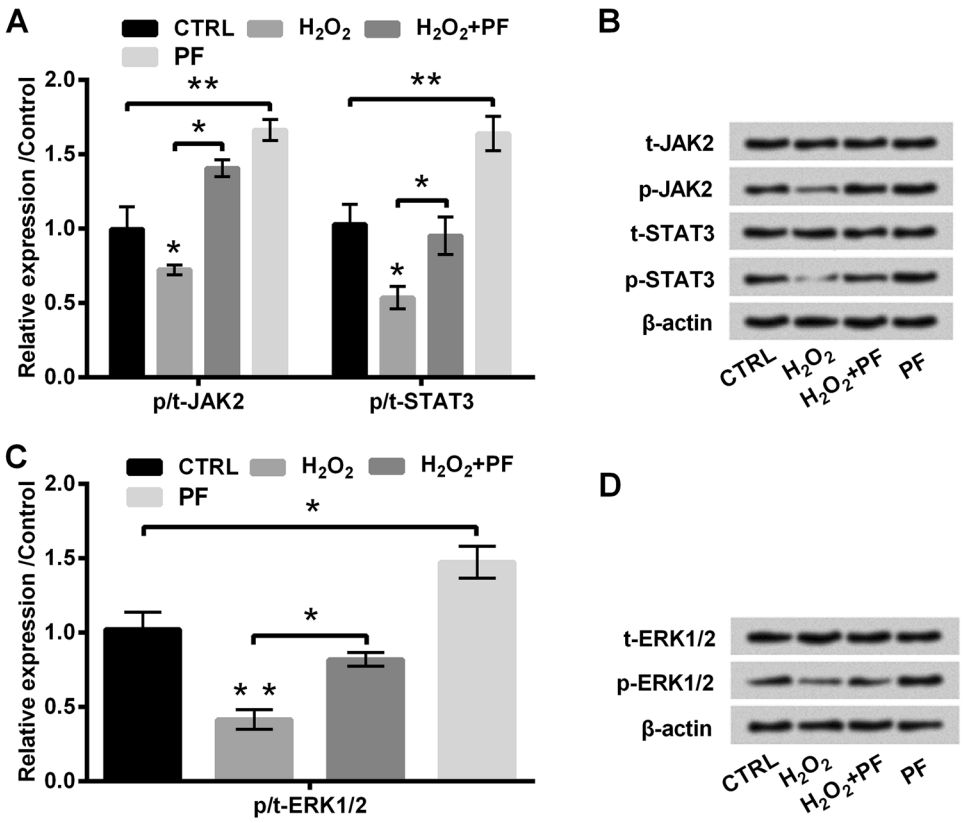


**Fig. 3** PF pretreatment promoted activation of JAK2/STAT3 and ERK1/2 pathways in $H_2O_2$-induced HT-22 cells. **a**, **b** PF treatment dramatically increased the expressions of proteins participated in JAK2/STAT3 pathway including p/t-JAK2 and p/t-STAT3. Besides, $H_2O_2$ treatment markedly reduced the expressions of p/t-JAK2 and p/t-STAT3, while PF pretreatment remarkably attenuated $H_2O_2$-triggered inhibitory effects. **c**, **d** PF treatment notably increased the expression of p/t-ERK1/2 which involved in ERK1/2 pathway. Besides, $H_2O_2$ treatment remarkably suppressed the expression of p/t-ERK1/2, while PF pretreatment markedly attenuated this inhibitory effect. *CTRL* control, *PF* Paeoniflorin, *JAK2* Janus kinase 2, *STAT3* signal transducer and activator of transcription 3, *ERK1/2* extracellular signal regulated kinase 1/2. $^{*}P<0.05$; $^{**}P<0.01$.

process of PF attenuating $H_2O_2$-triggered oxidative damage, we overexpressed miR-135a in HT-22 cells ($P < 0.01$, Fig. 5a). Results demonstrated that miR-135a mimic markedly reversed the promotion effects on cell viability induced by PF pretreatment ($P < 0.05$, Fig. 5b), as well as the promotion effect on CyclinD1 expression and the inhibitory effect on p53 expression triggered by PF pretreatment (both $P < 0.05$, Fig. 5c, d). In addition, we found that miR-135a mimic markedly reversed PF pretreatment-triggered suppression effect on cell apoptosis ($P < 0.05$, Fig. 5e), as well as the inhibitory effects on the expressions of Cleaved-caspase-3 and Cleaved-PARP (both $P < 0.01$, Fig. 5f, g). Furthermore, in $H_2O_2$ + PF exposure group, miR-135a mimic also significantly eliminated PF pretreatment-triggered suppression effect on ROS production ($P < 0.05$, Fig. 5h). These observations illustrated that PF pretreatment may achieved

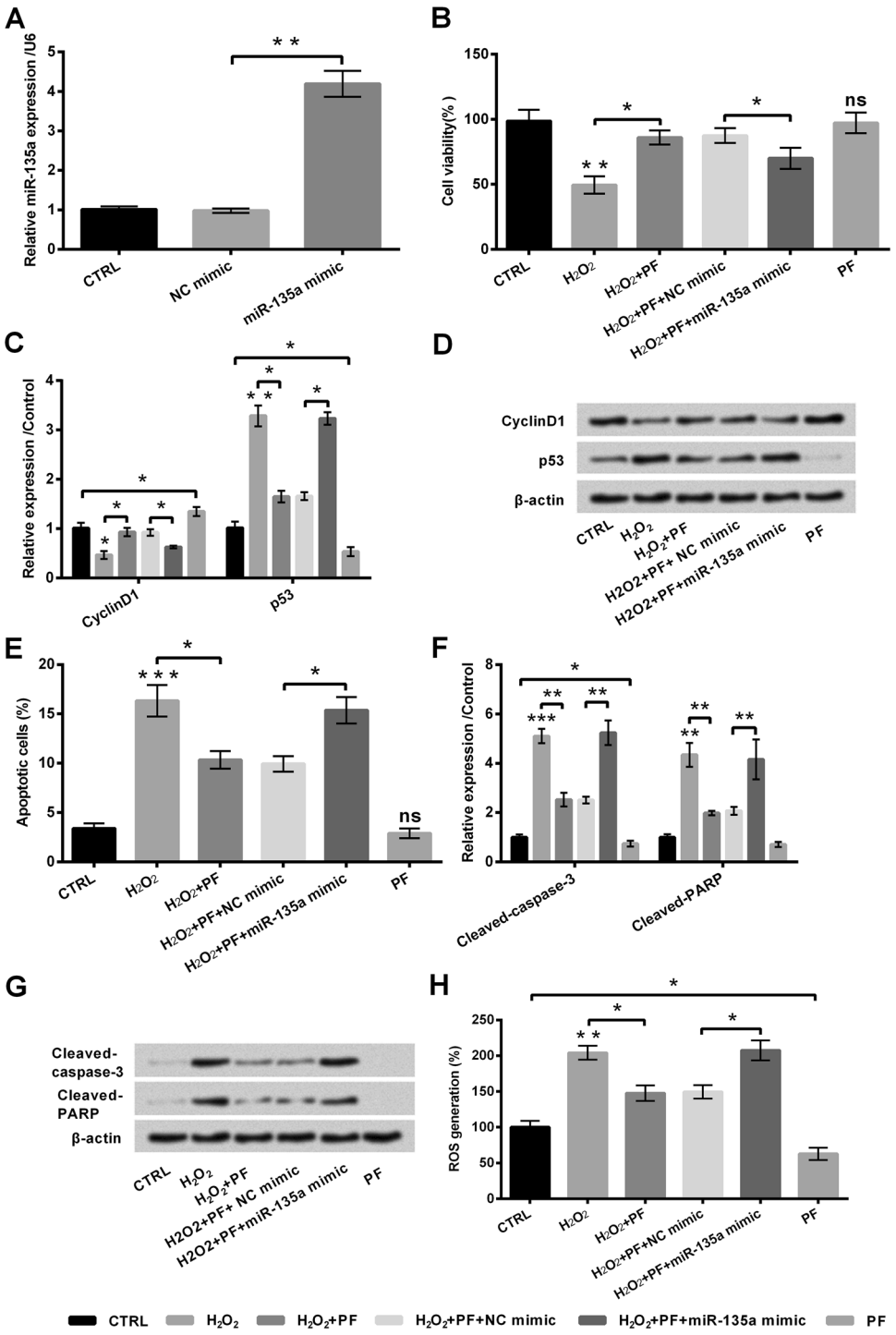


**Fig. 5** PF pretreatment alleviated $H_2O_2$-induced oxidative injury in HT-22 cells by reduction of miR-135a. **a** miR-135a was successfully overexpressed in HT-22 cells. **b** miR-135a overexpression notably revered PF pretreatment-triggered promotion effect on cell viability. **c**, **d** miR-135a mimic remarkably abolished PF pretreatment-triggered promotion effect on CyclinD1 expression and the inhibitory effect on p53 expression. **e** miR-135a mimic notably reversed the inhibitory effect on cell apoptosis. **f**, **g** miR-135a mimic remarkably abolished the inhibitory effects on the expressions of Cleaved-caspase-3 and Cleaved-PARP. **h** miR-135a mimic markedly reversed the inhibitory effect on ROS generation. *CTRL* control, *PF* Paeoniflorin, *PARP* poly-ADP-ribose polymerase, *ROS* reactive oxygen species, *ns* no significance, *miR-135a* microRNA-135a, *NC* negative control. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

its protective roles in HT-22 cells through decreasing the expression of miR-135a.

### PF Pretreatment Promoted Activation of JAK2/STAT3 and ERK1/2 Pathways Through Down-Regulation of miR-135a

To explore the underlying mechanism of PF regulation, we determined the expressions of key factors involved in JAK2/STAT3 and ERK1/2 pathways after miR-135a overexpression. Results suggested that miR-135a mimic notably abolished the promotion effects on the expressions of p/t-JAK2 ($P < 0.05$), p/t-STAT3 ($P < 0.05$) and p/t-ERK 1/2 ($P < 0.05$) triggered by PF pretreatment (Fig. 6a–d). These results indicated that PF pretreatment might promote activation of JAK2/STAT3 and ERK1/2 pathways through decreasing the expression of miR-135a.

## Discussion

Previous studies have shown that PF possessed neuroprotective effects on hippocampal dysfunction animal models [21], and could ameliorate cognitive dysfunction in diabetic rats [22], therefore, it might be a promising traditional Chinese medicine (TCM) for AD treatment. To address this speculation, we firstly constructed an injury model of AD in HT-22 cells via $H_2O_2$ treatment and then investigated the protective effects of PF on oxidative injured HT-22 cells induced by $H_2O_2$ treatment, and attempted to explore the potential regulating mechanisms. Our results specified that PF could alleviate the oxidative damage induced by $H_2O_2$ in HT-22 cells via promoting the activation of JAK2/STAT3 and ERK1/2 pathways through down-regulating the expression of miR-135a.

To elucidate how PF exerts its therapeutic effects on $H_2O_2$-induced oxidative injured HT-22 cells, we investigated the potential mechanisms. Herein, our results showed that PF itself treatment had no significant influence on cell viability. Whereas, following trials suggested that PF had significantly alleviated $H_2O_2$-induced oxidative damage and exhibited a certain protective effect on HT-22 cells, including increasing cell viability, decreasing cell apoptosis and declining ROS generation. Our study was in accordance with a previous study performed in osteoblastic MC3T3-E1 cells which demonstrated that PF provided a protective effect against methylglyoxal-induced cell damage via reducing oxidative stress [23]. We further explored the underlying mechanism(s). CyclinD1 is an important oncogene protein that regulates cell cycle and promotes G1/S phase transition [24]. p53 plays important role in stress responses, and it can trigger cell cycle arrest and apoptosis in response to

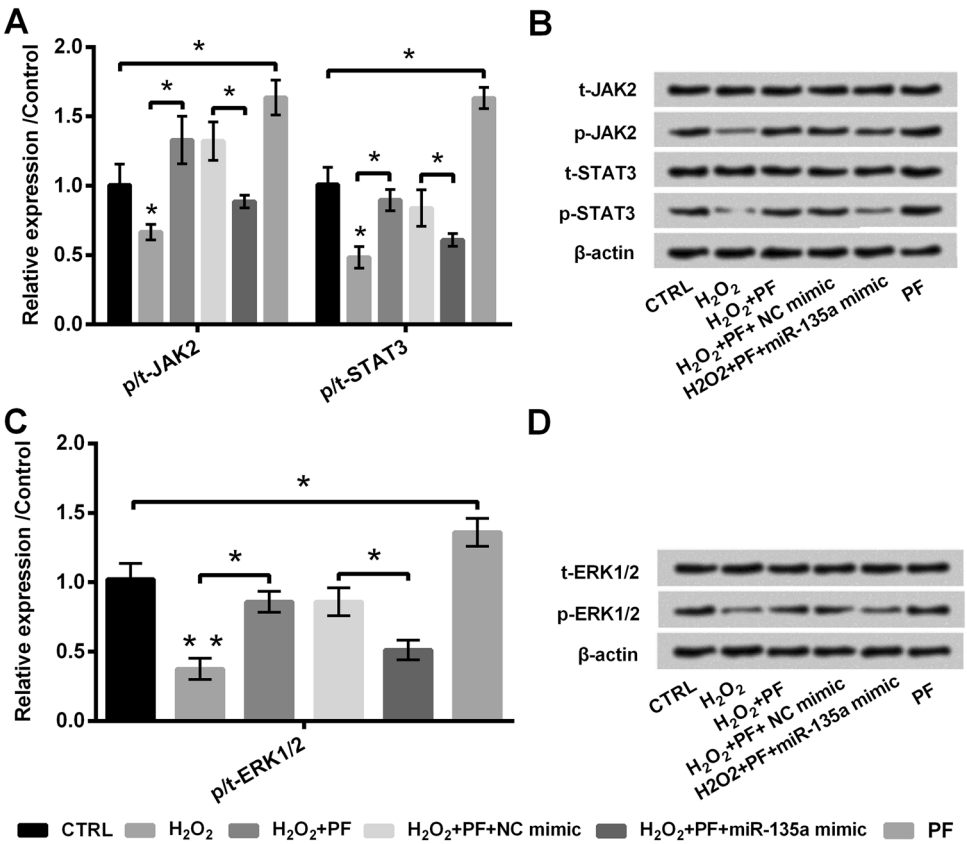


**Fig. 6** PF pretreatment promoted activation of JAK2/STAT3 and ERK1/2 pathways in $H_2O_2$-induced HT-22 cells by decreasing miR-135a expression. **a**, **b** miR-135a mimic markedly abolished PF pretreatment-triggered promotion effects on the expressions of p/t-JAK2 and p/t-STAT3 which was involved in JAK2/STAT3 pathway. **c**, **d** miR-135a mimic remarkably reversed PF pretreatment-triggered promotion effect on p/t- ERK1/2 expression which participated in ERK1/2 pathway. *CTRL* control, *PF* Paeoniflorin, *JAK2* Janus kinase 2, *STAT3* signal transducer and activator of transcription 3, *ERK1/2* extracellular signal regulated kinase 1/2, *NC* negative control. $^*P < 0.05$; $^{**}P < 0.01$

diverse stresses, including hypoxia, oxidative stress, DNA damage, ribonucleotide depletion and nutrient starvation [25]. Our results demonstrated that PF treatment markedly enhanced the expression of CyclinD1 protein and down-regulated the expression of p53 protein which further verified that PF was capable of alleviating the inhibitory effect of $H_2O_2$ on cell proliferation. However, the roles of PF in cancers were completely contrary to this, which exhibited as inhibiting cell proliferation [8–11]. Our research verified that PF also had a protective effect on AD. Earlier studies verified that Cleaved-caspase 3 acted as an "actuator" to cleave structural and regulatory proteins in the cell, thereby enhanced apoptosis [26]. Caspase cleavage of the PARP protein to polypeptide indicates that the cell is undergoing apoptosis [27]. Flow cytometry showed that cell apoptosis was significantly reduced by PF treatment. Molecularly, the expression of Cleaved-caspase-3 and Cleaved-PARP were dramatically down-regulated by PF treatment. Moreover, accumulating studies clarified that oxidative stress was one of the pathogenic factors in the progression of AD [28, 29]. The imbalance between the generation and elimination of ROS resulted in oxidative stress, which may further lead to nervous system dysfunction and neuronal apoptosis [30, 31], leading to nervous system diseases such as AD. Herein, our experiments confirmed that PF treatment significantly decreased ROS generation induced by $H_2O_2$ treatment. This was in accordance with earlier studies clarifying that PF alleviated advanced oxidation protein product (AOPP)-induced oxidative injury through decreasing ROS production [32] and PF protected thymocytes by scavenging ROS to against irradiation-induced cell damage [33].

An increasing number of researches have indicated that TCM works by regulating the expression of miRNAs. Li et al. reported that PF inhibited doxorubicin-induced cardiomyocyte apoptosis via down-regulating the expression of miR-1 [34]. Besides, previous studies verified that PF inhibited cell proliferation and induced apoptosis by mediating miR-16 expression in human glioma cells [9, 10] and PF played the above roles via mediating the expression of miR-29b in multiple myeloma cells [8]. An literature review reported that miR-135a had been identified as one of the potential biomarkers for AD [17]. Therefore, we speculated that PF may play its role through regulating the expression of miR-135a. Interestingly, we observed that PF treatment could down-regulate the expression of miR-135a. To further confirm whether the effects of PF on $H_2O_2$-induced oxidative injury were achieved through down-regulation of miR-135a in HT-22 cells, we overexpressed miR-135a through cell transfection. Further experiments verified that overexpression of miR-135a can decline cell viability and enhance cell apoptosis. Moreover, overexpression of miR-135a down-regulated the expression of CyclinD1, while up-regulated the expressions of p53, Cleaved-caspase-3 and Cleaved-PARP. A study revealed that miR-135a was one of the ROS-regulated miRNAs [35]. Therefore, we suspected that overexpression of miR-135a may have an influence on ROS production. Our results verified that, ROS generation was markedly increased by miR-135a overexpression. Our study was in line with an earlier study proving that miR-135a overexpression can inhibit oxidative stress in atherosclerosis by decreasing ROS level [13]. These results suggested that PF alleviated $H_2O_2$-induced oxidative injury in HT-22 cells through down-regulating miR-135a expression.

It has been reported that the JAK2/STAT3 pathway might have a role in cognitive function and may have effects in the AD-relevant memory impairment [36]. Researchers previously verified that ERK1/2 pathway mediated neuroprotective effects against $H_2O_2$ insult [37, 38]. Considering these observations mentioned above, we examined the expressions of the key factors involved in JAK2/STAT3 and ERK1/2 pathway after PF treatment and cell transfection. Results indicated that the activation of these pathways induced by PF was abrogated by miR-135a mimic in $H_2O_2$-induced HT-22 cells. In other words, PF may achieve the activation of these pathways through decreasing the expression of miR-135a.

In conclusion, these results indicated that PF could alleviate $H_2O_2$-induced oxidative damage of HT-22 cells which might be achieved through mediating miR-135a expression and regulating JAK2/STAT3 and ERK1/2 pathways.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that there are no conflict of interest.

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