

Polygalasaponin F ameliorates middle cerebral artery occlusion-induced focal ischemia in rats through inhibiting TXNIP/NLRP3 signaling pathway

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Abstract

Polygalasaponin F (PGSF), an oleanane triterpenoid saponin extracted from *Polygala japonica*, has been demonstrated with neuroprotective effect. However, the therapeutic effects and mechanism of PGSF on focal ischemia remain unknown. In this study, we first established a rat model of focal ischemia using middle cerebral artery occlusion (MCAO) to evaluate the therapeutic effect of PGSF intervention and to investigate the impact of PGSF on the thioredoxin-interacting protein/NOD-, LRR-, and pyrin domain-containing protein 3 (TXNIP/NLRP3) inflammatory pathway. Secondly, brain neuron cells were isolated, and the cells received oxygen-glucose deprivation/reoxygenation (OGD/R) culture to establish the cell injury model *in vitro*. The mechanism of PGSF on the TXNIP/NLRP3 pathway was further validated. Our results showed that PGSF treatment reduced neurological scores, brain tissue water content and infarct volume and ameliorated the pathological changes in cerebral cortex in MCAO-induced focal ischemia rats. The TNF- α , IL-1 β and IL-6 levels decreased in MCAO-induced focal ischemia rats after PGSF treatment. Moreover, PGSF down-regulated the protein expressions of TXNIP, NLRP3, ASC, cleaved caspase-1, IL-1 β , and IL-18 in MCAO-induced focal ischemia rats. Meanwhile, PGSF treatment reduced the levels of apoptosis, ROS, inflammatory cytokine and TXNIP/NLRP3 pathway-related proteins (TXNIP, NLRP3, ASC, cleaved caspase-1, IL-1 β , and IL-18) in OGD/R-induced neuronal injury cells. Finally, PGSF treatment also inhibited the interaction between NLRP3 and TXNIP *in vitro*. In conclusion, our study demonstrated the therapeutic effects of PGSF on MCAO-induced focal ischemia rats. Moreover, the neuroprotective mechanism of PGSF on focal ischemia was associated with the inhibition of TXNIP/NLRP3 signaling pathway.

Introduction

Stroke is a group of conditions in which various risk factors cause the occlusion or rupture of intracerebral arteries, resulting in localized ischemic and hypoxic necrosis of the brain tissue with rapid corresponding neurological deficits (Barthels et al. 2020). It was reported that in 2019 there were 101 million prevalent cases of stroke worldwide; of these, 12.2 million were incident cases and 6.55 million were deaths caused by stroke (Global et al. 2021). Ischemic stroke was the most common type, accounting for 62.4% of the cases. As a disease that severe morbidity, mortality, recurrence, and disability worldwide, stroke has shown a trend of rejuvenation in recent years, which seriously affects the quality of life of patients and causes a heavy economic burden on their family and society (Ge et al. 2020).

The most effective treatment for acute ischemic stroke to date is acute brain tissue reperfusion, which mainly consists of intravenous thrombolysis and mechanical thrombectomy (Rabinstein et al. 2020). Tissue plasminogen activator (tPA), a thrombolytic agent, is the only drug currently approved by the U.S. Food and Drug Administration for the treatment of ischemic stroke (Barthels et al. 2020).

However, owing to the limited time window as well as the high treatment cost, many patients cannot receive the needed treatment to resolve the crisis. These procedures include prompt revascularization,

brain tissue reperfusion via intravenous thrombolysis and mechanical thrombectomy; consequently, they develop serious disabilities such as hemiplegia, speech disorders, and visual impairment. In addition, vascular reperfusion may lead to focal ischemia (Xu et al. 2021). There is currently no conclusive evidence from clinical trials show that neuroprotective agents are beneficial in acute ischemic stroke, and the treatment of stroke-induced speech and physical disabilities are worldwide challenges. Many studies have shown that traditional Chinese medicine (TCM) offers significant advantages in ameliorating post-stroke speech and physical disabilities. Salidroside inhibits neuroinflammatory responses after cerebral ischemia-reperfusion injury (CIRI) via the PI3K/Akt/HIF pathway (Wei et al. 2017). Total saponins from *Panax notoginseng* reduces brain injury by upregulating the expression of cyclic adenosine monophosphate (cAMP) and glycoprotein Ib platelet subunit alpha (GP1BA) proteins and downregulating the expression of platelet-activating factor (PAF) protein after middle cerebral artery occlusion (MCAO) (Xu et al. 2021). Breviscapine reduces oxidative damage in cerebral ischemia rats by upregulating the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) proteins (Guo et al. 2014). Buyang Huanwu Decoction regulates the endoplasmic reticulum stress-induced autophagy in the ischemic penumbra mice by upregulating the expression of growth arrest and DNA-damage-inducible 34 (GADD34) (Zhou et al. 2021). Elucidating the mechanism of action of TCM will help promote the application of TCM in the treatment of focal ischemia.

Polygala japonica Houtt., a plant of the genus *Polygala* in the family *Polygalaceae*, has anti-inflammatory, anti-bacterial, and anti-depressive properties (Fu et al. 2006). Polygalasaponin F (PGSF) is an oleanane triterpenoid saponin extracted from *Polygala japonica* Houtt. and has been shown with neuroprotective effect (Xie et al. 2020; Sun et al. 2012). In vitro studies have shown that PGSF can alleviate OGD / R-induced neuronal cell damage (Xie et al. 2020). In addition, intragastric administration of 100 µg / ml PGSF can improve brain tissue injury in the cerebral ischemia rat model (Zhou et al. 2019). PGSF could also inhibit the levels of the inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in oxygen glucose deprivation/reoxygenation (OGD/R)-stimulated BV-2 microglia (Gao et al. 2021); it also reduces the release of lipopolysaccharide (LPS)-induced inflammatory cytokines TNF-α and nitric oxide (NO), and decreases the expression of inducible nitric oxide synthase (iNOS) (Wei et al. 2014). These experiments demonstrate that PGSF has a strong inhibitory effect on neuroinflammation *in vitro*. More importantly, a study reported that PGSF reduces the size of cerebral infarct and decreases brain cell apoptosis in neonatal rats with hypoxic-ischemic brain injury (Liu et al. 2020). However, the effects and mechanisms of PGSF on focal ischemia remain unclear.

In this study, we first established a rat model of MCAO-induced focal ischemia to evaluate the therapeutic effect of PGSF and to investigate the effect of PGSF on the TXNIP/NLRP3 inflammatory pathway. Second, primary culture of brain neurons was performed, whose injury was induced via OGD/R, and PGSF intervention was applied to explore the detailed mechanism of action of PGSF.

Experimental Methods

Reagents and antibodies

Polygalasaponin F (PGSF; purity: 98%, cat. no.T3826) was obtained from Targetmol (United States). Naoxintong Capsule (NXT; cat. no.Z20025001) was obtained from Shaanxi Buchang Pharmaceutical Co., Ltd. (Shaanxi, China). DI-3-N-butylphthalide (NBP; cat. no.H20050299) was obtained from Shijiazhuang Pharmaceutical Group Enbipu Pharmaceutical Co., Ltd. (Shijiazhuang, China). CCK-8 Assay Kit (cat. no.KGA317) was purchased from KeyGEN BioTECH (Jiangsu, China). PAGE (cat. no.A1010), 1M Tris-HCL (cat. no.A1010), 1.5M Tris-HCL (cat. no.T1010) were all purchased from Solarbio (Beijing, China). DMEM was purchased from Gibco (United States). RIPA (cat. no.C1053) was obtained from Applygen (Beijing, China). BCA Protein Assay Kit (cat. no.E-BC-K318-M) was obtained from Elabscience (Shanghai, China). Rat IL-1 β (cat. no.MM-0047R1), IL-6 (cat. no.MM-0190R1), TNF- α (cat. no.MM-0180R1) enzyme-linked immunosorbent assay (ELISA) kit were obtained from Jiangsu Enzyme Immune Industrial Co., Ltd. (Jiangsu, China). Rabbit Anti-NSE (cat. no.bs-10445R) was obtained from BIOS (Shanghai, Chian). Goat Anti-Rabbit IgG CY3 (cat. no.As007) was obtained from ABclona (Wuhan, Chian). Rabbit anti-TXNIP (cat. no.DF7506), Rabbit anti-NLRP3 (cat. no.DF7438), Rabbit anti-Caspase-1 (cat. no.AF4022), Rabbit anti-ASC (cat. no.DF6304), Rabbit anti-IL-1 β (cat. no. AF5103), Rabbit anti-IL-18 (cat. no.DF6252), Rabbit anti- β -actin (cat. no.AF7018), Goat Anti-Rabbit IgG (H+L) HRP (cat. no.S0001), Goat Anti-Mouse IgG (H+L) HRP (cat. no.S0002) were obtained from affinity (Jiangsu, China). All other reagents used were of analytical grade.

Animals

The experimental animals were male Sprague Dawley (SD) rats, weighing 260–280 g, and were purchased from Sipeifu (Beijing) Biotechnology Co., Ltd., with animal license number of SCXK (Beijing) 2019-0010. The animals were acclimatized in a quiet environment for 1 week at room temperature (24°C–26°C) with a relative humidity of 40%–60% under a 12 h dark/light cycle. They were given plenty of clean water and free access to food. The experimental procedure was approved by the Animal Ethics Review Committee of the No.1 Affiliated Hospital of Yunnan University of Traditional Chinese Medicine (Approval Number: S2020-002), and the experimental process was conducted in accordance with the regulations on experimental animal management. All efforts were made to minimize the animals' suffering and the number of animals used. For euthanasia, the rats were deeply anesthetized with 1% pentobarbital 8 sodium (60 mg/kg i.p.).

Modeling of focal ischemia in rats

Modification of the method of Zea Longa (Ye et al. 2020) was used to generate the MCAO-induced focal ischemia model. First, the rat was anesthetized and fixed; then, an incision was made from the midline neck, where the right common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were carefully isolated. The proximal end of the CCA and the ECA were ligated. Simultaneously, the distal end of the ICA was clamped with a vascular clip. A small incision was made at the CCA, and a monofilament (0.38 $\pm$ 0.02 mm in diameter at the head end) was inserted in the direction of the ICA until the 19–20 mm mark of the monofilament entered the bifurcation of the ICA and CCA, causing focal ischemia in the right cerebral hemisphere. The monofilament was removed after 2 h of

ischemia to induce reperfusion injury. The rats in the Sham group were only subject to vessel separation (i.e., the monofilament was not inserted), and the rest of the operation was the same as that in the MCAO group. Measurements of relevant indicators were performed at 24 h after reperfusion. The criteria for successful modeling were as follows: rats awoke after surgery; displayed left-sided hemiparesis, which was prominent in the left upper limb; when the rat's tail was lifted and hung upside down, the left forelimb was flexed and adducted; the rat moved in circles to the left when crawling; and the right side was positive for the Horner's syndrome.

Animal grouping and drug administration protocol

Totally, 121 rats were used, 15 rats were randomly selected as the Sham group. The remaining 106 rats were subjected to MCAO. During the operation, 16 rats died. The 90 survived rats following MCAO operation were randomly divided into six groups: MCAO group, MCAO+DI-3-N-butylphthalide (NBP) group, MCAO+Naoxintong capsule (NXT) group, MCAO+PGSF low-dose group, MCAO+PGSF middle-dose group, and MCAO+PGSF high-dose group (n = 15 per group). The Sham and MCAO groups received intragastric administration of 1 mL/100 g of saline; the MCAO+NBP group received 90 mg/kg of NBP via intraperitoneal injection (Qin et al. 2019); the MCAO+NXT group received 500 mg/kg of NXT via intragastric administration (Wan et al. 2018); and the MCAO+PGSF low-dose, MCAO+PGSF middle-dose, and MCAO+PGSF high-dose groups received 35 mg/kg, 70 mg/kg, and 140 mg/kg PGSF, respectively, via intragastric administration (Longa et al. 1989). The drug was administered once-daily for 7 days after MCAO.

Neurological function and body weight examination

Seven days after MCAO, rats were scored for neurological function using the Zea Longa (Ye et al. 2020) five-grade 4-point scale scoring criteria, which was applied by a blinded observer: 0, no signs of neurological deficit; 1, inability to fully extend the opposite forepaw in the tail suspension test; 2, circling to the opposite side while crawling; 3, falling to the opposite side while crawling; and 4, no voluntary activity. Higher scores indicate more severe neurological damage. Besides, the body weight was measured at day 0, 1, 3 and 7 after MCAO.

Determination of water content in brain tissue

Seven days after MCAO, the rats were anesthetized, decapitated, and sacrificed, and the brains were quickly removed, placed on filter paper to absorb the surface water, and then placed on aluminum foil and for the measurement of the wet weight. Subsequently, they were dried in an incubator at a constant temperature of 80°C for 72 h until a constant weight was reached, which was considered the dry weight. The water content of brain tissue was calculated from the following equation (Tian et al. 2008): brain tissue water content = (wet weight – dry weight)/wet weight × 100 %.

Measurement of cerebral infarct volume

The rats were decapitated to obtain the brain, and the olfactory bulb, cerebellum, and lower brainstem were removed, frozen for 20 min at -20°C , cut into five coronal sections, each of 2 mm in thickness, starting from the frontal lobe to the posterior of the brain, and then immediately placed in 2 % 2,3,5-triphenyl tetrazolium chloride (TTC) solution, incubated at 37°C for 30 min at a constant temperature and protected from light, and finally fixed in 4 % paraformaldehyde solution at 4°C for 24 h. The normal area appeared deep red and the infarcted area appeared white. Images were taken using a digital camera and the red and white areas were measured using the Motic Images Plus 2.0 micrographic analysis system. The following equations were used to calculate the cerebral infarct volume (Khan et al. 2000):

$$\text{Mean infarct size (mm}^2\text{)} = (\text{anterior surface infarct area} + \text{posterior infarct area})/2$$

$$\text{Infarct volume (mm}^3\text{)} = \text{total infarct area} \times \text{brain slice thickness (2 mm)}$$

$$\text{Corrected cerebral infarct volume (mm}^3\text{)} = \sum \{ \text{non-ischemic volume} - (\text{hemispheric ischemic volume} - \text{measured cerebral infarct volume}) \}$$

$$\text{Cerebral infarct volume (\%)} = \text{corrected cerebral infarct volume} / (\text{healthy cerebral hemisphere volume} \times 2) \times 100 \%$$

Histopathological examination

At the end of the seven days after MCAO, the rats were killed, and the cerebral cortex was collected and fixed with formalin. The tissue sections were then cut into 7- μm thick sections after dehydration and enclosed in paraffin, and subsequently stained with hematoxylin and eosin (HE). The denatured cell index (DCI) was measured to evaluate the degree of cell damage as described previously (Wang et al. 2015). $\text{DCI} = \text{denatured cell number} / \text{total cell number} \times 100 \%$.

Primary brain neuron culture and identification

The brain tissue, taken from newborn SD rat within 24 h of birth, was isolated in pre-cooled Hank's balanced salt solution, cut into sections of 1 mm^3 , digested with 0.125 % trypsin, and resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (Guo et al. 2016). The suspension was filtered using a 70 μm sieve and planted on plates at a density of $1 \times 10^6/\text{mL}$ and inoculated in high-glucose DMEM containing 10% fetal bovine serum, 1 % sodium pyruvate, and 1 % Glutamax. After the cells were allowed to adhere for 4 h, the culture medium was changed to neurobasal maintenance medium containing 2 % B27 and 1 % Glutamax to maintain cell culture. Half the volume of medium was changed every 3 days. Cells were cultured until day 7. The cells with a purity of $\geq 92 \%$, as identified by immunofluorescence, were used in the experiments. The results are shown in Supplementary Material (**Figure S1**).

Construction of the OGD/R model

Primary brain neurons were washed three times with PBS, added to glucose-free DMEM, and placed in a tri-gas incubator (94 % N₂, 5 % CO₂, 1 % O₂, 37 °C); at the end of hypoxia, the glucose-free DMEM was replaced with DMEM containing 10 % fetal bovine serum, and the culture was continued at 37 °C with 5 % CO₂. The hypoxia/reoxygenation (H/R) times were 5 h/0.5 h.

Cell viability assay

After the treatments were applied to the cells, 10 % Cell Counting Kit-8 (CCK-8) solution was added to the cells and incubated at 37 °C for 2 h according to the kit instructions. The optical density (OD) for absorbance was measured at 450 nm on a microplate reader, and the relative viability of the cells was calculated relative to the control group treated with cell-free medium. The formula used for calculation was: cell viability value = (OD of experimental group – OD of blank control group)/(OD of control group – OD of blank control group) × 100 %.

Flow cytometry

After OGD/R modeling, cells were collected (density 1×10⁶/mL) and washed by PBS for 3 min twice, and 300 µL of pre-cooled 1× binding buffer was added to resuspend the cells. Then, 5 µL of Annexin V-FITC and 10 µL of PI were added, the cell solution was incubated for 10 min at room temperature and protected from light; finally, 200 µL of pre-cooled 1× binding buffer was added and mixed, and cell apoptosis was detected by NovoCyte™ flow cytometry. After the cells were treated, DCFH-DA (1:1000) was added, incubated at 37 °C for 20 min, and then washed three times with serum-free culture medium. Then, 1 mL PBS was added, centrifuged at 1,500 r/min for 5 min; the supernatant was discarded; and finally, 300 µL PBS was added to resuspend the cells on the machine to detect reactive oxygen species (ROS).

ELISA

Blood was obtained via the abdominal aorta, rested at room temperature, centrifuged at 3,000 r/min for 10 min, and serum was collected. The treated cells were centrifuged at 1,500 r/min for 3 min and the supernatant was retained. Indicator tests for inflammatory cytokines TNF-α, IL-1β, and IL-6 were performed in accordance with the kit instructions.

Western blotting

Total protein was extracted from cortical tissue on the infarct side or treated primary brain neurons with pre-cooled radioimmunoprecipitation assay (RIPA) lysate and centrifuged at 12,000 r/min for 10 min at 4 °C. The supernatant was aspirated into a new eppendorf (EP) tube, and the protein concentration was detected by using the bicinchoninic acid assay (BCA). After SDS-PAGE gel electrophoresis, membrane transfer, and blocking, TXNIP (1:1000 dilution), NLRP3 (1:1000 dilution), cleaved caspase-1 (1:1000 dilution), ASC (1:500 dilution), IL-1β (1:1000 dilution), IL-18 (1:1000 dilution), and β-actin (1:2000 dilution) primary antibodies were added, separately, and incubated overnight at 4 °C. The membranes were washed and horseradish peroxidase-labeled secondary antibody (1:10000 dilution) was added, incubated

for 2 h at room temperature, and washed again. Electrochemiluminescence solution was added to visualize the bands, and Image Pro software was used for quantitative analysis of the bands. The following formula was used to calculate the relative expression: relative protein expression = target protein gray value/internal reference protein gray value.

Co-immunoprecipitation (CO-IP)

The treated cells were placed in pre-cooled IP cell lysis buffer and lysed on ice for 30 min. After the samples were centrifuged at 16,000 r/min for 15 min at 4 °C, the supernatant was aspirated into a new EP tube. An aliquot of this sample was analyzed for levels of TXNIP, NLRP3, and a housekeeping protein. The remainder of the sample was subjected to IP experiments, i.e., TXNIP antibody was used to pull down the protein (with IgG was used as negative control) to obtain protein complexes, and then the protein complexes were analyzed by western blotting with the NLRP3 antibody.

Statistical analysis

The experimental results were analyzed using the SPSS statistical software (version 25.0). Data in the present study was presented as the mean $\pm$ standard deviation (SD). T-tests were used for data follow a normal distribution, whereas Kruskal–Wallis H tests were used for data that was not follow a normal distribution.

Results

Effect of PGSF on body weight in MCAO-induced focal ischemia rats

As indicated in Fig. 1, compared with the Sham group, the body weight was significantly decreased in the MCAO group at day 0, 1, 3 and 7. Compared with the MCAO group, the body weight was significantly increased in the MCAO + NXT group ($P < 0.01$), MCAO + NBP group ($P < 0.01$) and MCAO + PGSF high-dose group ($P < 0.01$) at day 3 and 7.

Effect of PGSF on neurological scores, brain tissue water content, infarct volume and pathological changes in MCAO-induced focal ischemia rats

As indicated in Fig. 2a, there was no neurological deficit in the Sham group rats, but a significantly higher neurological deficit score in the MCAO group ($P < 0.01$). Compared with the MCAO group, the neurological function score was significantly reduced in the MCAO + PGSF high-dose group ($P < 0.01$).

The water content in brain tissue in the MCAO group was significantly increased compared with the Sham group ($P < 0.01$). Compared with the MCAO group, water content in brain tissue was significantly reduced in the MCAO + PGSF high-dose group ($P < 0.01$) (Fig. 2b).

The TTC staining revealed that the brain tissue of normal rats was stained a deep red color and the infarcted brain tissue was stained white; there was no white infarcted area in the Sham group (Fig. 2c). Compared with the Sham group, the cerebral infarct size was significantly increased in the MCAO group ($P < 0.01$); then, compared with the MCAO group, the cerebral infarct size was significantly decreased in the MCAO + NXT group ($P < 0.01$), MCAO + NBP group ($P < 0.01$), MCAO + PGSF middle-dose group ($P < 0.01$), and MCAO + PGSF high-dose group ($P < 0.01$) (Fig. 2d).

HE staining showed that the morphology of neurons was normal, and no significant pathological changes could be observed in cerebral cortex in Sham group. Whereas irregularly arranged neurons and cellular swelling could be observed in MCAO group. Treatment of NXT, NBP and PGSF ameliorated the pathological changes in MCAO-induced focal ischemia rats. (Fig. 2e). Likewise, the DCI was higher in MCAO group than that in the Sham group and was lower in MCAO + NXT group ($P < 0.01$), MCAO + NBP group ($P < 0.01$), MCAO + PGSF middle-dose group ($P < 0.01$), and MCAO + PGSF high-dose group ($P < 0.01$) than that in MCAO group (Fig. 2f).

Effect of PGSF on inflammatory cytokine levels and TXNIP/NLRP3 pathway-related proteins in MCAO-induced focal ischemia rats

As indicated in Fig. 3, the levels of TNF- α ($P < 0.01$), IL-1 β ($P < 0.01$), and IL-6 ($P < 0.01$) were significantly increased in the brain tissue of the infarcted side of the rats in the MCAO group compared to that in the Sham group; compared with the MCAO group, the levels of TNF- α were significantly decreased in the MCAO + NBP and MCAO + PGSF high-dose groups ($P < 0.01$); the levels of IL-1 β were significantly decreased in the MCAO + NBP, MCAO + PGSF low-dose, MCAO + PGSF middle-dose, and MCAO + PGSF high-dose groups ($P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.01$); the levels of IL-6 were significantly increased in the MCAO + NXT group ($P < 0.01$); and the levels of IL-6 were significantly decreased in the MCAO + NBP group and the MCAO + PGSF high-dose group ($P < 0.01$, $P < 0.01$).

As indicated in Fig. 4, the results showed that the levels of TXNIP ($P < 0.01$), NLRP3 ($P < 0.01$), ASC ($P < 0.01$), cleaved caspase-1 ($P < 0.01$), IL-1 β ($P < 0.01$), and IL-18 ($P < 0.01$) were significantly increased in the brain tissue from the infarcted side of the rats in the MCAO group compared with those in the Sham group; compared with the MCAO group, the levels of TXNIP ($P < 0.01$), NLRP3 ($P < 0.01$), IL-1 β ($P < 0.01$), IL-18 ($P < 0.01$), and ASC ($P < 0.01$, $P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.01$) were significantly decreased in the MCAO + NXT, MCAO + NBP, MCAO + PGSF low-dose, MCAO + PGSF middle-dose, and MCAO + PGSF high-dose groups; and the levels of cleaved caspase-1 ($P < 0.01$) were significantly decreased in the MCAO + NXT, MCAO + NBP, MCAO + PGSF middle-dose, and MCAO + PGSF high-dose groups.

Effect of PGSF on cell viability, apoptosis, and ROS in a model of OGD/R-induced neuronal injury

To investigate the effect of different doses of PGSF on the viability of normal primary brain neurons, cells were treated with PGSF at concentrations of 0.1, 1, 10, and 100 μ M, individually (Zhou et al. 2021). The CCK-8 method results showed that there was no significant effect on cell viability at PGSF concentrations of 0.1, 1, and 10 μ M compared with the normal cells, but that cell viability was significantly inhibited

when the PGSF concentration was 100 μM ($P < 0.05$) (Fig. 5a). Therefore, concentrations of 0.1, 1, and 10 μM were selected for subsequent experiments investigating the effect of PGSF on the OGD/R cell model.

The CCK-8 method results showed that cell viability was significantly lower in the OGD/R group than in the control group ($P < 0.01$); compared with the OGD/R group, the cell viability in the OGD/R + PGSF (1 μM) group ($P < 0.01$), and OGD/R + PGSF (10 μM) group ($P < 0.01$) was significantly increased and tended to increase with an increase in drug concentration (Fig. 5b). Therefore, a PGSF concentration of 10 μM was selected for subsequent experiments.

The flow cytometry results showed that the level of apoptosis was significantly higher in the OGD/R group than in the control group ($P < 0.01$); compared with the OGD/R group, the level of apoptosis was significantly lower in the OGD/R + PGSF group ($P < 0.01$). At the same time, compared with the control group, ROS levels were significantly higher in the OGD/R model cells ($P < 0.01$); moreover, compared with the OGD/R model group, ROS levels were significantly lower in the OGD/R + PGSF group ($P < 0.05$) (Fig. 5c).

Effect of PGSF on inflammatory cytokine levels and TXNIP/NLRP3 pathway-related proteins in OGD/R-induced neuronal injury in model cells

The ELISA results showed that the TNF- α , IL-1 β , and IL-6 levels in the cells of the OGD/R group were significantly higher than in the control group ($P < 0.01$); moreover, the TNF- α , IL-1 β , and IL-6 levels in the OGD/R + PGSF group were significantly decreased ($P < 0.05$, $P < 0.01$, $P < 0.01$) compared with that of the OGD/R group (Fig. 6).

The western blotting results showed that the levels of TXNIP, NLRP3, ASC, cleaved caspase-1, IL-1 β , and IL-18 were significantly higher in the OGD/R group than those in the control group ($P < 0.01$); and levels of TXNIP ($P < 0.01$), NLRP3 ($P < 0.01$), ASC ($P < 0.01$), cleaved caspase-1 ($P < 0.01$), IL-1 β ($P < 0.01$), and IL-18 ($P < 0.01$) in the OGD/R + PGSF group were significantly lower than in the OGD/R group (Fig. 7).

The CO-IP results showed that there was interaction between NLRP3 and TXNIP. The interaction of NLRP3 and TXNIP was enhanced in the OGD/R group relative to the control group ($P < 0.01$), and the interaction of NLRP3 and TXNIP was reduced in the OGD/R + PGSF group compared with the OGD/R group ($P < 0.01$) (Fig. 8).

Discussion

In this study, the MCAO was used to generate a rat model of focal ischemia, which simulates the pathological process of ischemic stroke. Our results showed that in this model, the results were consistent with other articles that reported neurological deficit scores ranging from 3–4, brain infarct volumes ranging from 30–40%, and brain tissue water content ranging from 80–90% (Liu et al. 2019; Sha et al. 2019); thus, the model was successfully generated. PGSF significantly improved neurological deficits and reduced cerebral edema in MCAO-induced focal ischemia rats while reducing the volume of

cerebral infarcts. In addition, to better evaluate the therapeutic effect of PGSF on focal ischemia, we selected NBP, a neuroprotective drug against ischemic brain injury, and NXT, a proprietary TCM patent medicine with neuroprotective effects against acute ischemic stroke, as positive control drugs, respectively. Previous animal studies and clinical trials have shown that these drugs have neuroprotective effects (Zhu et al. 2018; Liu et al. 2016). Our results showed that the high-dose of PGSF intervention was superior to the positive control drug groups in improving neurological function and cerebral edema, with no significant difference between the PGSF low-, middle-, and high-dose groups and the positive drug groups intervention in reducing the volume of cerebral infarcts. Together, this suggests the significant therapeutic effect of PGSF in treating ischemic stroke.

We then examined the effect of PGSF on the inflammatory response in MCAO-induced focal ischemia rats. PGSF significantly downregulated the levels of inflammatory cytokines IL-1 β , TNF- α , and IL-6. Within hours of the onset of cerebral ischemia, microglia and astrocytes become activated, leading to the production of cytokines and chemokines, as well as leukocyte infiltration (Jayaraj et al. 2019); for example, the expression of cytokines such as IL-1 β , TNF- α , and IL-6 is rapidly upregulated, producing and exacerbating the inflammatory response after stroke (Yang et al. 2019; Zhu et al. 2021). IL-1 β is the main pro-inflammatory cytokine, and previous studies have found that IL-1 β increases infarct size after MCAO (Touzani et al. 2001). TNF- α is also an important pro-inflammatory cytokine in the pathology of ischemic stroke; its serum levels increase within 6 h and persist for 10 days after stroke (Zaremba et al. 2001). Anti-TNF α treatment significantly improves endothelial cell necrosis, attenuates disruption of the blood–brain barrier, and improves stroke outcome (Chen et al. 2019). IL-6 is a pleiotropic cytokine that can be identified within 12–72 h of acute stroke onset and is a marker of inflammation in acute ischemic brain injury (Pawluk et al. 2020).

Next, we investigated the effect of PGSF on the TXNIP/NLRP3 signaling pathway. The results showed that PGSF significantly downregulated the levels of NLRP3, ASC, cleaved caspase-1, IL-1 β , and IL-18. The NLRP3 inflammasome is one of the most studied members of the inflammasome family, which contains NLRP3, ASC, and pro-caspase-1 (Davis et al. 2011; Buscetta et al. 2020; Zhang et al. 2021). NLRP3 inflammasome is the most generally studied inflammasome, which might regulate the discharge of IL-1 β and IL-18. several studies have shown that NLRP3 inflammasome is related to the spread of system diseases and chronic inflammatory and metabolic diseases, as well as coronary artery disease, I/R injury, and Parkinson's disorder (Mangan et al. 2018). NLRP3 belongs to the NLRs macromolecule family. The structure of most NLRs consists of 3 components: N-terminal peptidase enlisting region, pyrin domain (PYD), acidic trans-activation domain, or baculovirus anti-apoptotic macromolecule repeat domain (Wang et al. 2020). When NLRP3 recognizes pathogens and intracellular danger signals, it oligomerizes and binds to the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and recruits Caspase-1 precursors, which form aggregates of NLRP3 inflammasomes and induce Caspase-1 aggregation and autocatalysis, IL-1 β and L-18. Inflammatory factors are eventually activated and released into extracellular space by cleavage from inactive precursors (Li et al. 2021). The NLRP3 inflammasome plays a dominant role in the early stages of stroke and can trigger an inflammatory cascade response after ischemic stroke (Franke et al. 2021). After the onset of ischemic stroke, NLRP3

inflammasomes are activated; activated NLRP3 recruits ASC and binds to pro-caspase-1 to form an inflammasome and activated caspase-1, which cleaves pro-IL-1 β and pro-IL-18 in cells, prompting them to form mature IL-1 β and IL-18 with that subsequently released from the cells, which in turn exacerbates the inflammatory response by the neurons.

Similarly, our results confirm that PGSF significantly downregulates TXNIP expression. TXNIP is an important regulatory protein in NLRP3 inflammasome activation. Under physiological conditions, TXNIP in cells is tightly bound to TRX, forming the TXNIP-TRX complex, and is in a state of inhibition. The onset of ischemic stroke leads to oxidative stress and ROS formation, which prompt TXNIP to dissociate from TRX and bind to NLRP3, leading to NLRP3 inflammasome activation followed by interaction with ASC and release of pro-caspase-1 which induces formation of proinflammatory cytokine release (Zhou et al. 2010;Zhang et al. 2015).

In addition, in this study, primary brain neurons were used to generate an *in vitro* OGD/R model to simulate the neuronal injury process during ischemic stroke. Our results showed that approximately 30–50% of cells were apoptotic in the OGD/R cell model, which was consistent with other articles (Si et al. 2020), indicating that the model was successfully established. We found that PGSF significantly increased the viability of cells in the OGD/R-induced neuronal injury model, inhibited apoptosis, decreased ROS levels, and downregulated inflammatory cytokine levels. Meanwhile, western blotting experiments confirmed the inhibitory effect of PGSF on the TXNIP/NLRP3 pathway in the OGD/R-induced neuronal injury model, which is consistent with the results of *in vivo* studies. Subsequently, CO-IP experiments were employed to further validate whether PGSF could alter the interaction between NLRP3 and TXNIP in the OGD/R-induced neuronal injury model. CO-IP is a commonly used method to detect protein interactions. Our results showed that PGSF inhibits the interaction between NLRP3 and TXNIP, suggesting that inhibition of the NLRP3/TXNIP pathway activation is one of the important targets through which PGSF acts to improve focal ischemia.

In conclusion, our study first used *in vivo* and *in vitro* experiments to demonstrate that PGSF could reduce the inflammatory response in MCAO-induced focal ischemia rats through inhibiting the activation of the TXNIP/NLRP3 pathway in neurons. Our study enriched the scientific evidence for the pharmacological mechanism of PGSF on focal ischemia. Currently, inhibiting neuroinflammation through regulating NLRP3 signaling pathway provides new thoughts for the treatment of focal ischemia (Milner et al. 2021). Our study could also provide the scientific evidence in illustrating the neuroinflammatory inhibiting mechanism of natural products. There are also some limitations in this study. Only neurological scores were measured to evaluate the effect of PGSF on the outcome of MCAO-induced focal ischemia. Body temperature is also an important index in evaluating the infarct progression (He et al. 1999). More indicators such as body temperature should be tested to further access the effect of PGSF on infarct progression. Furthermore, many studies have revealed the neuroprotective effect of PGSF. The brain protective and behavioral improving effect of PGSF will be studied in our future study by testing the influence of PGSF on behavioral outcomes such as long-term sensorimotor deficits and cognitive deficits (at least 4 weeks after I/R injury).

Declarations

Ethics approval and consent to participate

All methods were carried out in accordance with relevant guidelines and regulations. The animal experiments were carried out in compliance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and approved by the Animal Ethics Review Committee of the No.1 Affiliated Hospital of Yunnan University of Traditional Chinese Medicine (Approval Number: S2020-002)

Consent for publication

Not applicable.

Availability of data and materials

The raw data and materials supporting the conclusions of this article will be made available by the corresponding authors, without undue reservation.

Conflict of Interests

The authors declare that they have no conflicts of interest.

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Contributions

Yao Chen and Han-zhou Li carried out the experiments and manuscript writing. Yang Yan, Lei Feng provided experiments and manuscript writing help. Xi-Xing Fang and Jiao-Bao Liao experiments, performed data analysis and result interpretatio. Jie Zhao, Xiao-Chi Xin and Shu-Quan Lv, supervised the experiments. Wei-Bo Wen and Huan-Tian Cui provided ideas and technical guidance for the whole work. All authors contributed to the article and approved the submitted version.

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Figures

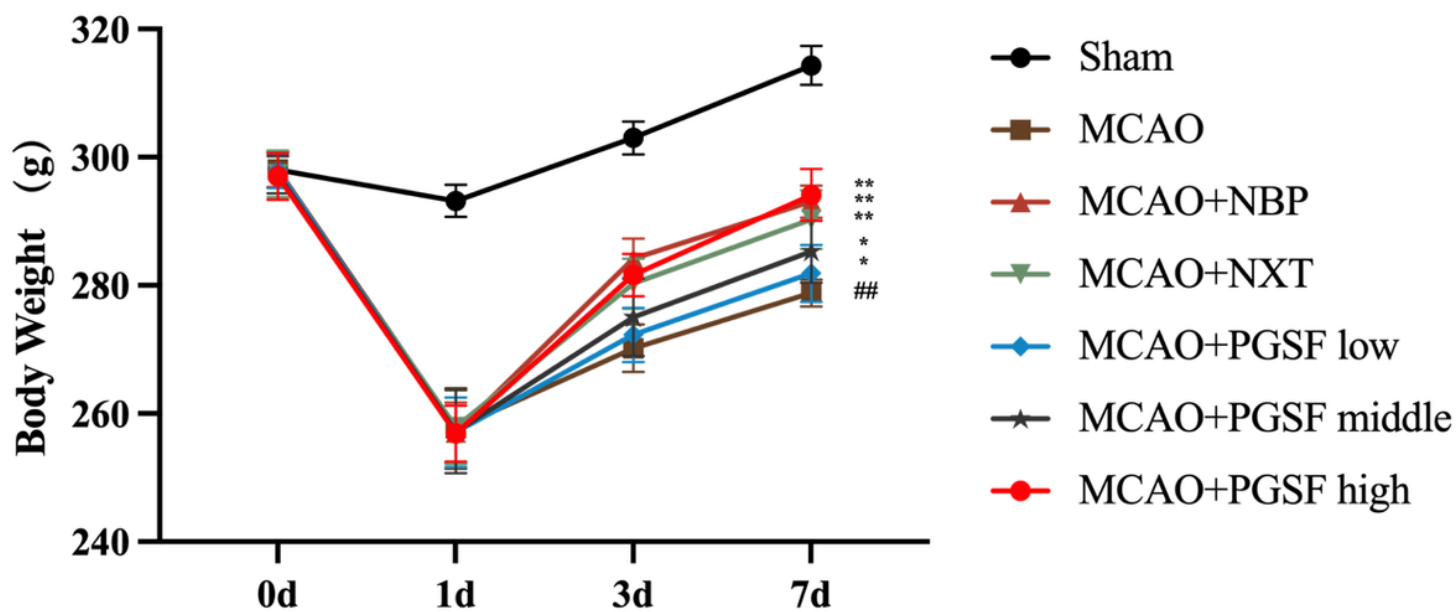


Figure 1

Effect of PGSF on body weight in MCAO-induced focal ischemia rats. PGSF treatment reduced the body weight loss by MCAO operation (n=15 per group). Data are presented as the mean $\pm$ SD. ## $P < 0.01$ compared to the control group. * $P < 0.05$ compared to the experimental model group. ** $P < 0.01$ compared to the experimental model group.

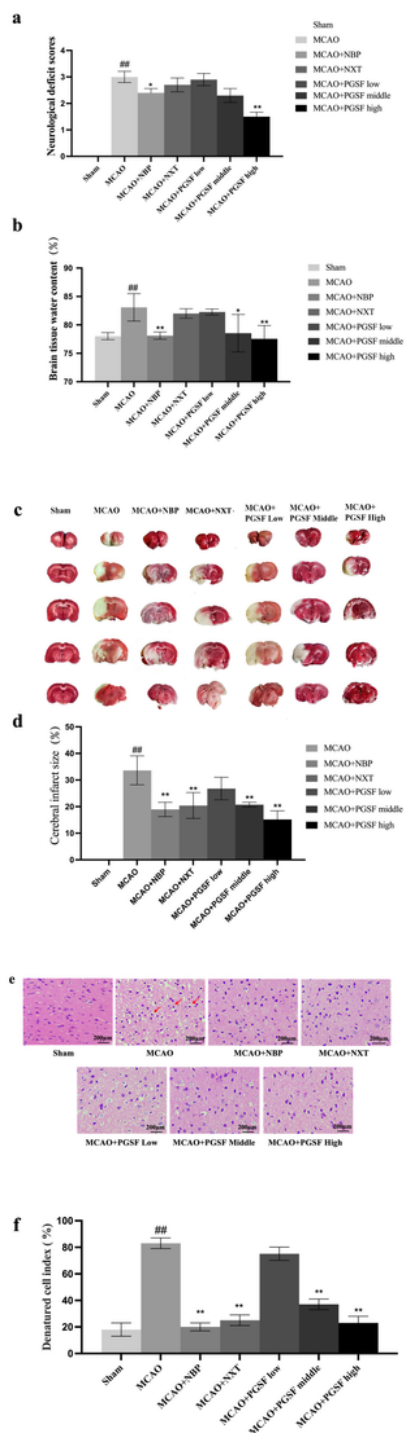


Figure 2

PGSF treatment reduced neurological scores, brain tissue water content, infarct volume and improved pathology changes in MCAO-induced focal ischemia rats. (a) PGSF treatment decreased the neurological scores in MCAO-induced focal ischemia rats (n=15 per group). (b) PGSF treatment decreased the brain tissue water content in MCAO-induced focal ischemia rats (n=15 per group). (c d) PGSF treatment decreased the infarct volume in MCAO-induced focal ischemia rats (n=6 per group). (e,f) HE staining

indicated that PGSF treatment improved the pathology changes in cerebral cortex (200×) (e) and decreased DCI (f). Red arrows indicated the cellular swelling (n=9 per group). Data are presented as the mean ± SD. ## $P<0.01$ compared to the control group. * $P<0.05$ compared to the experimental model group. ** $P<0.01$ compared to the experimental model group.

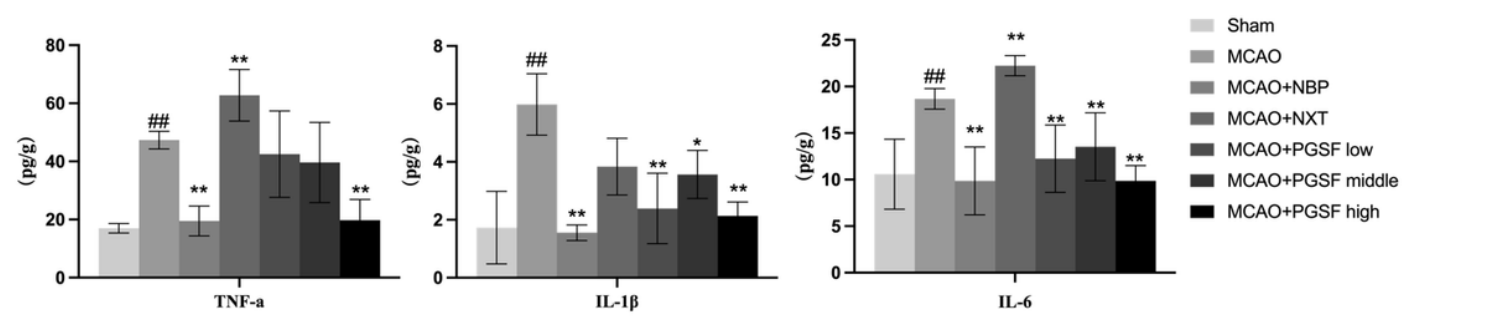


Figure 3

PGSF treatment decreased the levels of pro-inflammatory cytokines in MCAO-induced focal ischemia rats (n=15 per group). Data are presented as the mean ± SD. ## $P<0.01$ compared to the control group. * $P<0.05$ compared to the experimental model group. ** $P<0.01$ compared to the experimental model group.

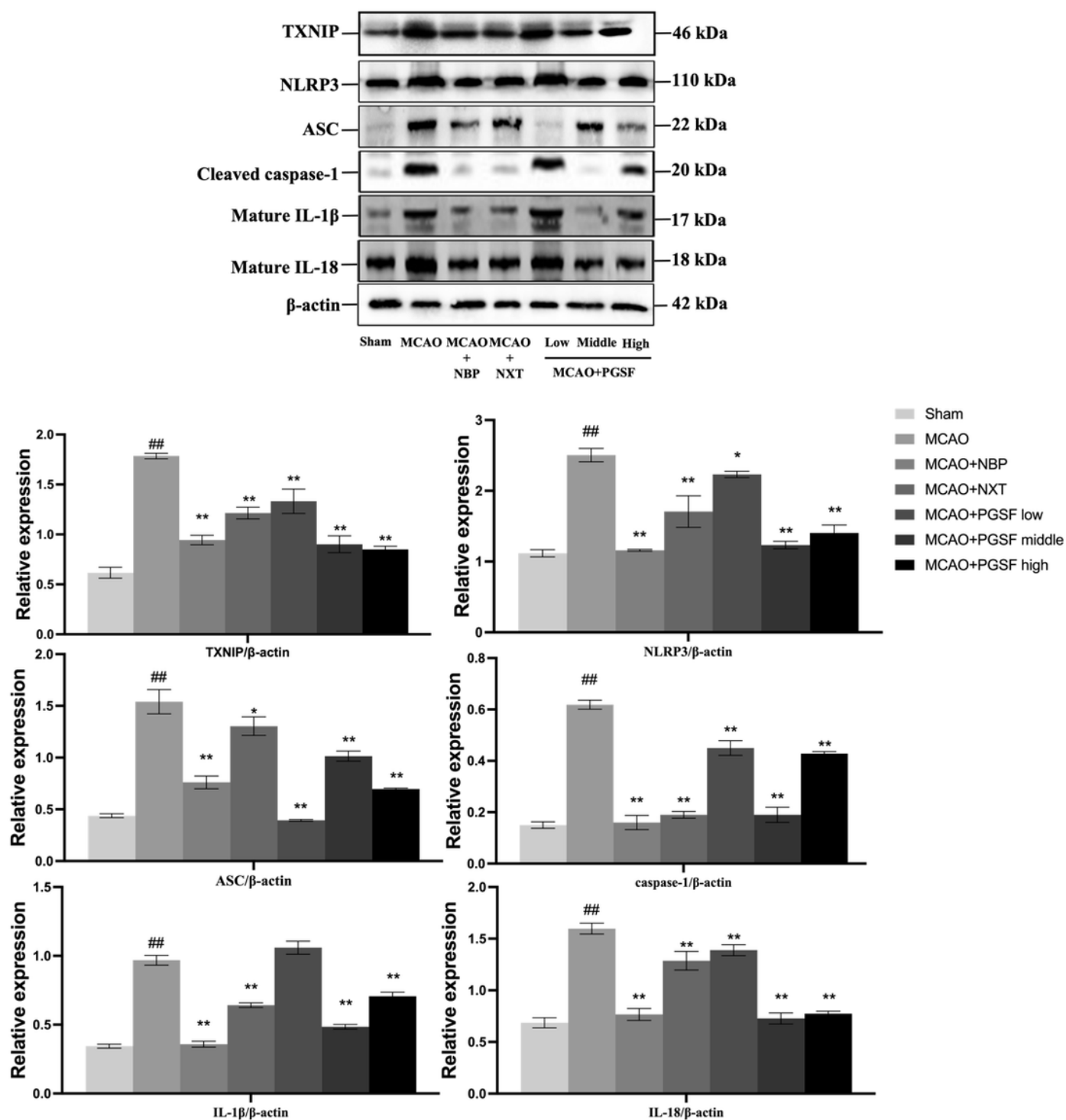


Figure 4

PGSF treatment decreased the levels of TXNIP/NLRP3 pathway-related proteins in MCAO-induced focal ischemia rats. PGSF down-regulated the protein expressions of TXNIP, NLRP3, ASC, cleaved caspase-1, IL-1 β , and IL-18 in MCAO-induced focal ischemia rats (n=3 per group). Data are presented as the mean $\pm$ SD. ## P < 0.01 compared to the control group. * P < 0.05 compared to the experimental model group. ** P < 0.01 compared to the experimental model group.

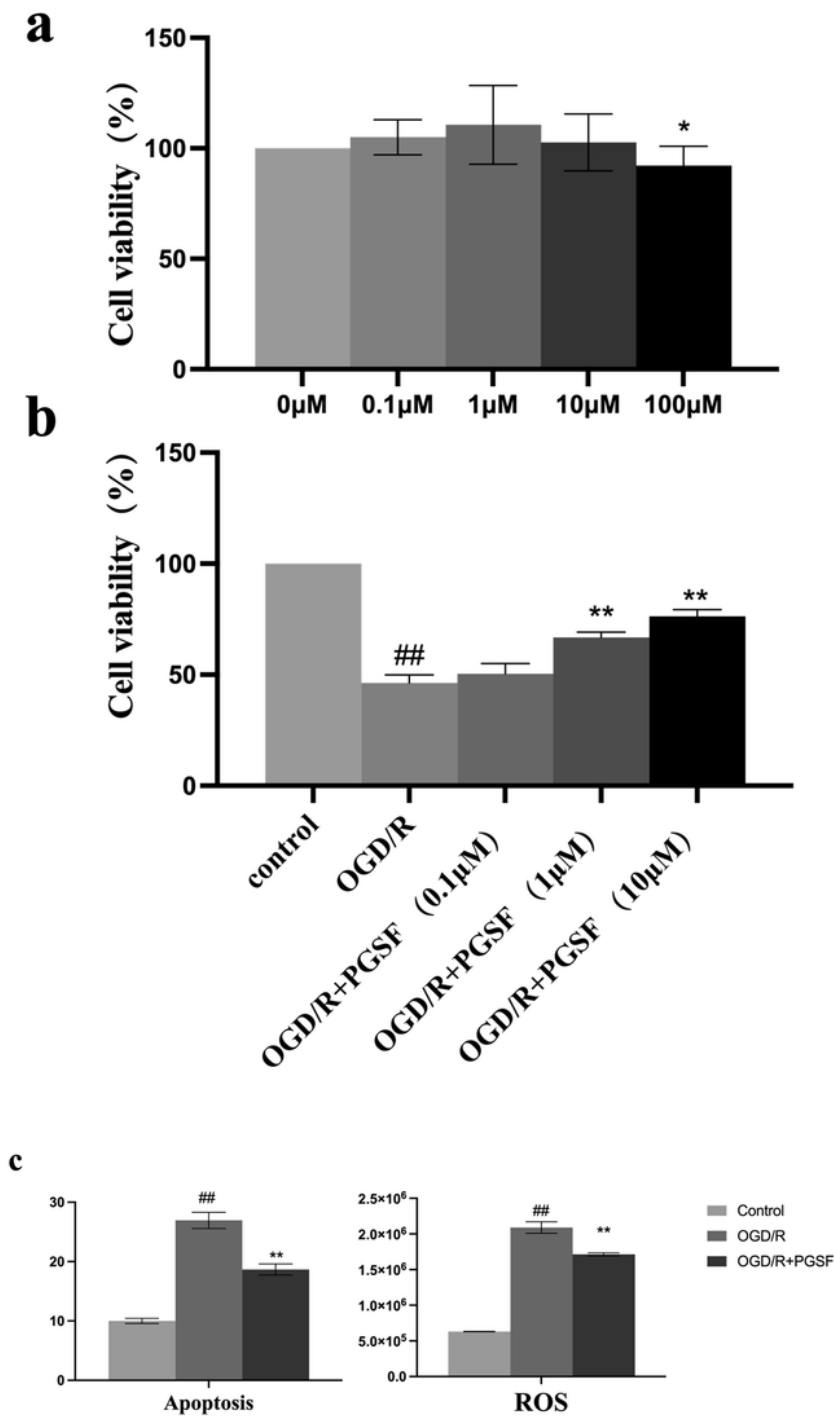


Figure 5

Effect of PGSF on cell viability, apoptosis, and ROS in a model of OGD/R-induced neuronal injury. (a,b) Cell viability was detected with a CCK-8 assay. (c) cell apoptosis and ROS assay were performed to evaluate whether PGSF was involved in the OGD/R-induced neuron injury (n=3 per group). Data are presented as the mean $\pm$ SD. ## $P < 0.01$ compared to the control group. * $P < 0.05$ compared to the experimental model group. ** $P < 0.01$ compared to the experimental model group.

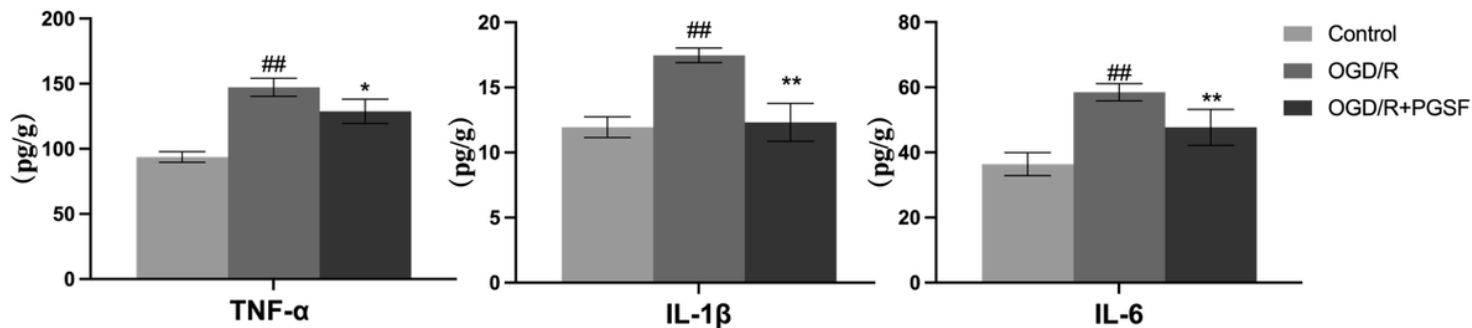


Figure 6

PGSF treatment decreased the levels of pro-inflammatory cytokines in OGD/R-induced neuronal injury in model cells (n=6 per group). Data are presented as the mean ± SD. ^{##} *P*<0.01 compared to the control group. ^{*} *P*<0.05 compared to the experimental model group. ^{**} *P*<0.01 compared to the experimental model group.

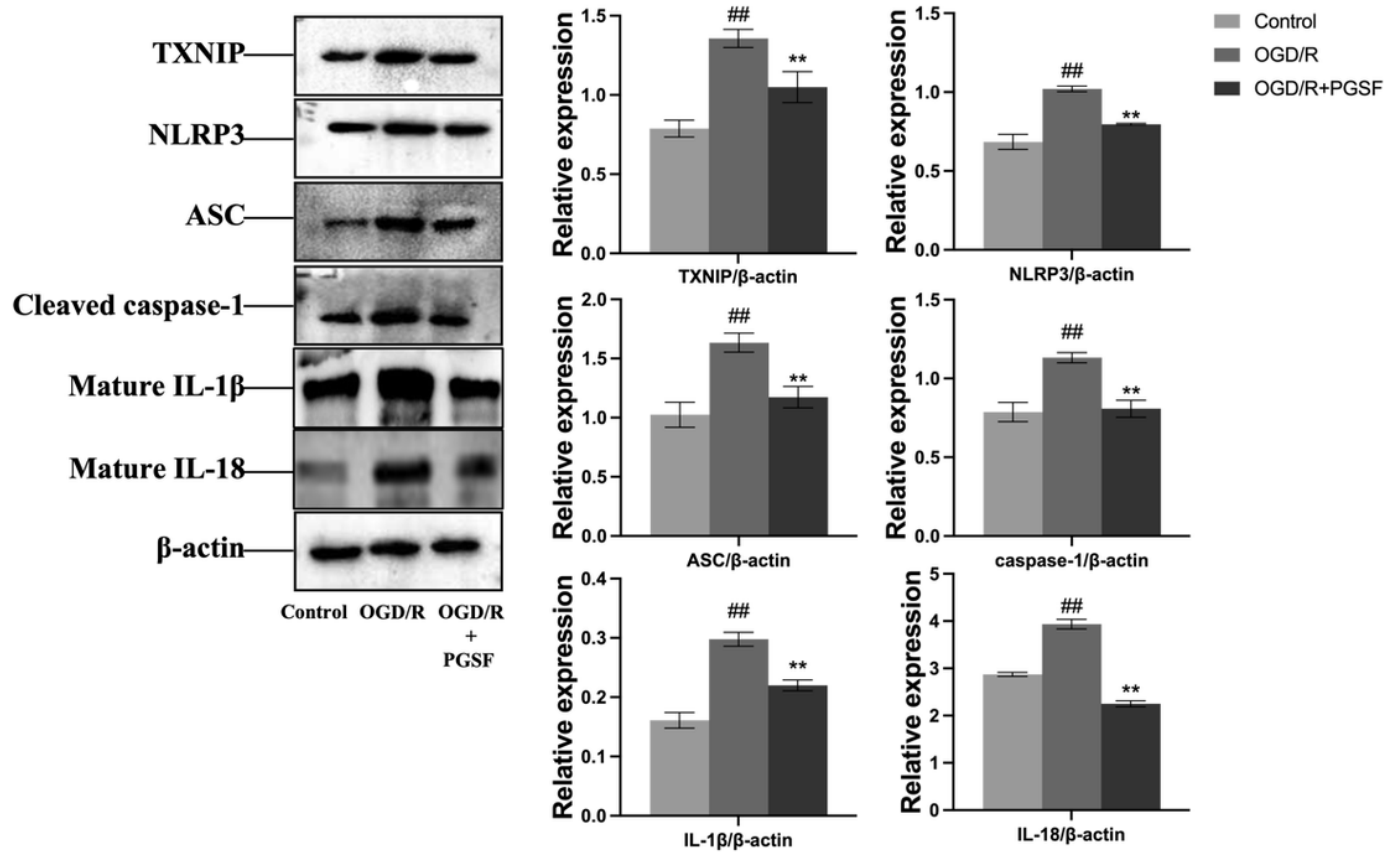


Figure 7

PGSF treatment decreased the levels of TXNIP/NLRP3 pathway-related proteins in OGD/R-induced neuronal injury in model cells. PGSF down-regulated the protein expressions of TXNIP, NLRP3, ASC, cleaved caspase-1, IL-1β, and IL-18 in OGD/R-induced neuronal injury in model cells (n=3 per group). Data

are presented as the mean $\pm$ SD. ## $P < 0.01$ compared to the control group. ** $P < 0.01$ compared to the experimental model group.

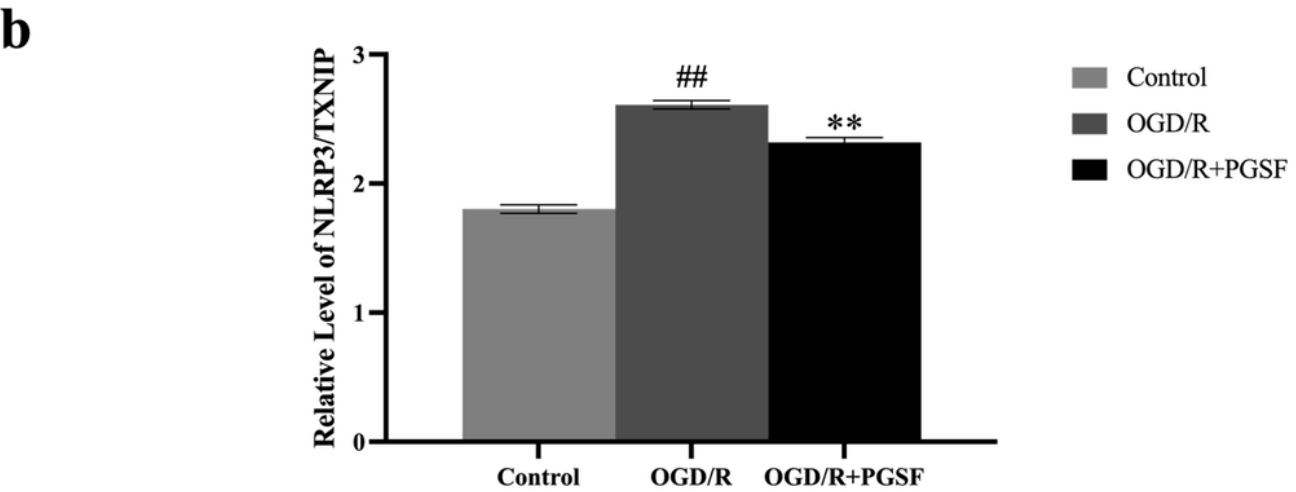
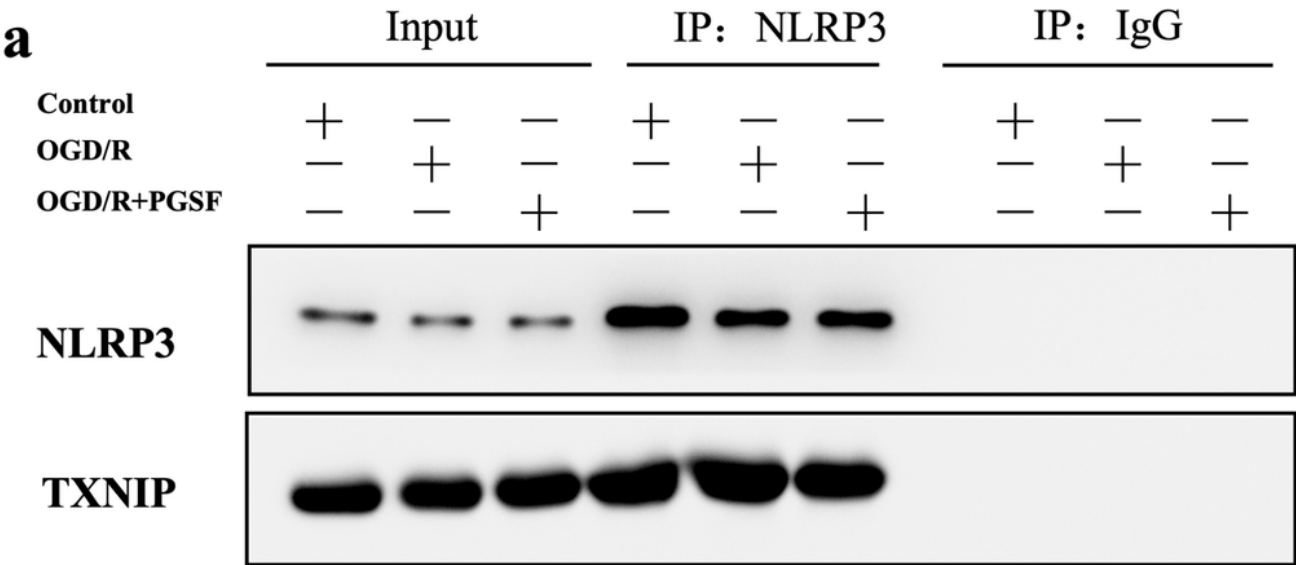


Figure 8

CO-IP analysis demonstrated that there was interaction between NLRP3 and TXNIP. (a)PGSF treatment decreased the interaction of NLRP3 and TXNIP. Each assay was conducted for three times.(b)Grayscale

analysis for ratio of NLRP3/TXNIP in b was performed using Image J software (n=6 per group). Differences between groups were evaluated by t-test. Data are presented as the mean $\pm$ SD. ## $P < 0.01$ compared to the control group. ** $P < 0.01$ compared to the experimental model group.

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