Research paper

Chrysophanol postconditioning attenuated cerebral ischemia-reperfusion injury induced NLRP3-related pyroptosis in a TRAF6-dependent manner

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\textbf{Abstract}
Individuals who suffer from post-CA (cardiac arrest) brain injury experience higher mortality and more severe functional disability. Neuroinflammation has been identified as a vital factor in cerebral ischemia-reperfusion injury (CIRI) following CA. Pyroptosis induces neuronal death by triggering an excessive inflammatory injury. Chrysophanol possesses robust anti-inflammatory features, and it is protective against CIRI. The purpose of this research was to assess the effect of Chrysophanol postconditioning on CIRI-induced pyroptotic cell death, and to explore its underlying mechanisms. CIRI was induced in rats by CA and subsequent cardiopulmonary resuscitation, and PC12 cells were exposed to oxygen-glucose deprivation/reoxygenation (OGD/R) to imitate CIRI in vitro. It was found that post-CA brain injury led to a notable cerebral damage revealed by histopathological changes and neurological outcomes. The existence of pyroptosis was also confirmed in in vivo and in vitro CIRI models. Moreover, we further confirmed that Chrysophanol, the main bioactive ingredient of Rhubarb, significantly suppressed expressions of pyroptosis-associated proteins, e.g., NLRP3, ASC, cleaved-caspase-1 and N-terminal GSDMD, and inhibited the expression of tumor necrosis factor receptor-associated factor 6 (TRAF6). Furthermore, NLRP3 overexpression neutralized the neuroprotection of Chrysophanol postconditioning, suggesting that pyroptosis was the major neuronal death pathway modulated by Chrysophanol postconditioning in OGD/R. Additionally, the neuroprotection of Chrysophanol postconditioning was also abolished by gain-of-function analyses of TRAF6. Finally, the results demonstrated that Chrysophanol postconditioning suppressed the interaction between TRAF6 and NLRP3. Taken together, our findings revealed that Chrysophanol postconditioning was protective against CIRI by inhibiting NLRP3-related pyroptosis in a TRAF6-dependent manner.

\textbf{1. Introduction}
Cardiac arrest (CA) is one of the common causes of death worldwide (Shao et al., 2014; Mozaffarian et al., 2016). Cases who successfully received resuscitation after CA still have high rates of in-hospital mortality and disability, mainly due to neurological impairments induced by irreversible brain injury (Neumar et al., 2008). Although new and effective therapeutic techniques are continuously developed, the survival rate of patients suffering from post-CA brain injury (PCABI) still remains extremely low (Stock et al., 2019). Thus, early diagnosis and treatment of neurological impairments after CA are vital in clinical settings.

Although the possible underlying mechanism of PCABI has not been fully explored, neuroinflammation has been widely recognized because of its essential role (Ousta et al., 2022; Liu and McCullough, 2013). As a kind of mediated necrosis, pyroptosis refers to cell death caused by cell lysis related to inflammation (Frank and Vince, 2019). The prominent features of pyroptosis are closely related to the rapid plasma-membrane lysis and the leakage of a large number of pro-inflammatory intracellular products (Frank and Vince, 2019; Man et al., 2017). Emerging evidence has demonstrated that activation of NOD-like receptor pyrin domain-containing protein 3 (NLRP3)-related inflammasomes promoted the transformation of procaspase-1 into caspase-1, and subsequently enhanced maturation of IL-1β and IL-18, and GSDM (cleaved gasdermin)
family, eventually leading to pyroptosis (Li et al., 2021). Recent studies have reported that NLRP3 inflammassome activation-mediated pyroptosis pathway is associated with cerebral ischemia-reperfusion injury (CIRI) (Liu et al., 2021; Xiao et al., 2021; Xu et al., 2021). Nevertheless, few studies have concentrated on the exact role and significance of pyroptosis in PCABI.

Chrysophanol, known as a natural anthraquinone, possesses several pharmacological effects, including anti-oxidative, anti-inflammatory (Ma et al., 2021), anti-fibrotic (Yong et al., 2020), and anti-cancer activities (Park et al., 2018). The data from in vitro or in vivo studies have proved that Chrysophanol, an important bioactive component of Rhubarb, is a potential candidate to inhibit inflammation by regulating macrophage activities and suppressing the expression of pro-inflammatory cytokines through nuclear factor-κB (NF-κB) signaling pathway (Wen et al., 2018; Wen et al., 2021). Furthermore, Chrysophanol showed a neuroprotective effect, which inhibited mitochondrial autophagy (Cui et al., 2022) and attenuation of endoplasmic reticulum (ER) stress response (Zhao et al., 2018a). However, it has still remained elusive whether the protective property of Chrysophanol is involved in NLRP3-related pyroptosis during CIRI.

As cerebral ischemia is rarely preventable, numerous studies have concentrated on the intervention in the reperfusion period. A great number of studies have recently reported that potent pharmacological agents applied at the onset of reperfusion could significantly reduce the incidence of CIRI (Hwang et al., 2017; Grewal et al., 2019). Hence, whether Chrysophanol post-treatment has robust protective effects against CIRI remains to be elucidated. Increasing evidence suggested that Chrysophanol has markedly attenuated cerebral injury after focal CIRI, which may be partly attributed to its anti-oxidative stress (Zhao et al., 2018b) or anti-inflammatory effects (Zhao et al., 2016). Moreover, Chrysophanol may alleviate the ischemic brain damage via inhibiting the activation of NLRP3 inflammasomes (Zhang et al., 2014). Therefore, it was hypothesized that the protective roles of Chrysophanol in CIRI was associated with suppressed NLRP3-related pyroptosis.

Tumor necrosis factor receptor-associated factor 6 (TRAF6), which belongs to TRAF protein family, has been recently reported to participate in inflammatory responses through different intracellular signaling pathways (Zhang et al., 2015; Wei et al., 2021). For instance, TRAF6 promoted the activation of NF-κB, which was subsequently translocated to nuclei, resulting in increased transcriptions of pro-inflammatory cytokines (Huang et al., 2021). In addition, TRAF6-associated pro-inflammatory activity contributed to the progression of myocardial (He et al., 2021) and hepatic (Liu et al., 2020) ischemia-reperfusion injury. Of note, suppression of TRAF6 significantly decreased the pyroptosis-associated inflammatory reactions (Yan et al., 2020), while overexpression of TRAF6 remarkably promoted pyroptosis through the caspase-1/3 signaling pathways (Wei et al., 2021). Hence, TRAF6 is characterized as a novel pro-inflammatory regulator. However, the effect of TRAF6 on NLRP3-related pyroptosis in CIRI is still unclear.

Hence, the purpose of this research was to assess the effect of Chrysophanol post-treatment on CIRI-elicted pyroptotic cell death, and to explore its underlying mechanisms.

2. Materials and methods

2.1. Experimental animals

Experimental procedures were conducted according to the international ethics guidelines after approved by the Ethics Committee of Xiangya Hospital of Central South University (Changsha, China). Male Sprague-Dawley rats, weighing 300–350 g, were obtained from Silaikejingda Co., Ltd. (Changsha, China). Rats were maintained under optimal conditions (50% humidity, twenty-five centigrade degree, and a 12:12 light/dark cycle), and fed appropriately for seven days prior to the experiments.

2.2. Experimental model

A total of 156 SPF (specific-pathogen free)-level male adult Sprague-Dawley rats were classified into three groups randomly. CA/CPR (cardiopulmonary resuscitation) was not induced in the sham-operated group (30 rats), while all other surgical steps were carried out. We constructed the CA/CPR model based on the literature with some modifications (Chang et al., 2020). Rats were anesthetized with an intraperitoneal injection of 2% sodium pentobarbital (4 mg/100 g; B5646; Apexbio, Shanghai, China), and the trachea was intubated with a 14-gauge cannula. The right carotid vein was cannulated by a PE-50 tube for central venous pressure monitoring and medical administration. The right carotid artery was cannulated with a PE-25 tube, which was connected to a pressure transducer (BL-4208; Chengdu Taimeng Software Co., Ltd., Chengdu, China) for real-time monitoring of blood pressure. Cardiac rhythm was monitored using a three-lead ECG (electrocardiogram) with electrocardiograph leads inserted subcutaneously into the chest wall. The core body temperature was maintained at 37 °C by a thermistor-controlled heat lamp. The incision sites were infiltrated with 0.5% ropivacaine (AstraZeneca, Cambridge, UK) for postoperative analgesia. CA was induced using vecuronium (2 mg/kg; Mylan Institutional LLC., Rockford, IL, USA), followed by disconnection of the ventilator and obstruction of the intubation tube. Ten minutes later, CPR was commenced, which included administration of 10 μg/ml epinephrine (100 μg/kg; Hefeng Co., Ltd., Beijing, China) and 0.5 ml 5% sodium bicarbonate (Huiyinbi Co., Ltd., Shanghai, China) through the jugular vein. Chest compression was performed, followed by ventilation using a volume-controlled small animal ventilator (provided by Beijing Zhong Shi Di Chuang Science and Technology Development Co. Ltd., Beijing, China). Additional dose of sodium bicarbonate was administered 5 min after CPR according to the arterial blood pressure. Blood gas analysis was carried out 10 min before and after ROSC (return of spontaneous circulation), and it was defined as an “organized cardiac rhythm with a mean arterial pressure of ≥50 mmHg, which is sustained continuously for at least 10 min.” (Ocak et al., 2020a). Rats without ROSC following standard CPR for 15 min were defined as resuscitation failure.

After CA/CPR, survivors were randomly divided into CA group and CA + Chrysophanol (CA + CHR) group, respectively. Chrysophanol (purchased from National Institutes for Food and Drug Control, Beijing, China) with a purity of >98% was dissolved in 0.9% NaCl with 1% DMSO (dimethyl sulfoxide) and 1% Tween-80 before the treatment. Chrysophanol (10 mg/kg) was intraperitoneally administered at 24 h after CA/CPR, lasting for 7 consecutive days. The dosage of Chrysophanol was chosen in the present study based on previous studies (Zhao et al., 2018a; Zhao et al., 2018b; Cui et al., 2022). Rats in the sham and CA groups were given a corresponding volume of saline with 1% DMSO and 1% Tween-80 that would serve as vehicle. Body weight and survival rates were evaluated before or at 3 and 7 days after intraperitoneal administration, respectively.

To establish a CIRI model in vitro, PC12 cells were exposed to OGD/R (oxygen-glucose deprivation/reoxygenation) to imitate CIRI. Briefly, PC12 cells were washed with PBS (phosphate-buffered saline), and then, cultured in a glucose-free medium. The PC12 cells were cultured in an incubator chamber (3110; Thermo Fisher Scientific, Waltham, MA, USA) under conditions of 95% N2 and 5% CO2 for 6 h, and then, were discarded to culture in a medium containing glucose for one day under conditions of 95% O2 and 5% CO2 (Chen et al., 2022). For in vitro studies, PC12 cells were incubated with Chrysophanol, an important component of Rhubarb. The cells were firstly added into stock solution, then diluted to different concentrations (0, 10, 50, 100, 200 μM) and subsequently subjected to re-oxygenation to induce pharmacological post-conditioning. After 4 cycles of Chrysophanol administration (60-min Chrysophanol treatments with an interval of 60 min), the Chrysophanol-containing culture medium was replaced with a normal medium. The treatment with MCC950 (10 μM) or necrosulfonamide (10 μM, NSA) was performed, which was similar to Chrysophanol treatment,
to clarify the role of pyroptosis in OGD/R. Each experiment was carried out at least three times.

2.3. Neurological deficit score

Each rat was scored according to the mNSS (modified neurological severity score) (Lv et al., 2020) determined by charts at 1, 3, and 7 days after CA/CPR. Scores ranged from 0 (normal) to 18 (most severe neurological damage).

2.4. MWM (Morris water maze) test

The steps of MWM test were consistent with the previous literature (Wang et al., 2021). In order to assess memory and spatial learning, we placed the animals in a circular pool which was 1.6 m in diameter and 60 cm in height. A platform of 12 cm was immersed in the water (2.0 cm below the water), and the temperature was maintained at 25 ± 1 °C. The swimming path and escape latency were recorded automatically. Into a quiet room with visual cue decoration the water maze containing four quadrants was put. We placed the animals into the maze at diverse beginning sites, which were trained to identify a platform without signs. The maximum trial duration was 120 s for each trial, and four cycles of trials were performed daily. One day after the terminal training session, the probe trial was performed. We removed the platform, and the memory of the animals concerning the platform sites was assessed. We recorded the time spent on the target quadrant and the number of times that each rat crossed over the exact location of the former platform. After the test, the animals were killed for histological examination.

2.5. Determination of brain water content

The brain tissues (n = 5, per group) were dried at 105 °C for one day to detect the dry weight. Then, brain water content (%) was defined as follows: brain water content (%) = [(wet weight-dry weight)/wet weight] × 100%.

2.6. Histological examination

After the observation period, a certain brain tissue was selected from each group to assess the histological changes of hippocampus, because the CA1 area is sensitive to ischemia following cerebral injury. The brain tissues were fixed in paraformaldehyde solution, and then, embedded into paraffin. Sections (5 μm) were formed, and deparaffinized, followed by H&E (hematoxylin and eosin) staining, Nissl staining, and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining. Nissl-positive and TUNEL-positive cells are counted. Sections were examined randomly under a light microscope (magnification, 100 × and 400 ×, respectively) by an investigator who was blinded to grouping. At a high magnification, the number of positive cells in 4 fields of view was randomly counted, and the average number of positive cells was calculated.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Before harvesting the brain tissues, blood collection needles and tubes were used to collect blood from the left ventricle, which was placed at −4 °C overnight, followed by centrifugation at 3000 rpm for 10 min. The pale yellow supernatant was aspirated with a pipette, and the serum was aliquoted into 2 ml cryotubes, and stored at −80 °C for further experiment. An ELISA kit was used to detect IL-1β and IL-18 according to the manufacturer’s instructions (Wuhan Huamei Biotech Co., Ltd., Wuhan, China). At a wavelength of 450 nm, the OD (optical density) value of each rat serum sample was measured and recorded after the above-mentioned steps. The “Curve Expert” drawing software was used to plot a standard curve, and the above OD value was introduced into the regression equation for the sample concentration. To calculate the concentration of the diluted sample, multiplication was conducted by the inverse of dilution factor.

2.8. RT-qPCR (quantitative reverse transcription polymerase chain reaction)

Total RNA was isolated from brain samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and PC12 cells were harvested. Then, 5 μm RNA was utilized to reversely transcribe into cDNA using the Revert Aid First Strand cDNA Synthesis kit (provided by Invitrogen; cat. no. K1622), according to the manufacturer’s instructions. The 2^ΔΔCt method was used to determine the relative mRNA expressions. Table 1 shows the sequences of primers.

2.9. Cellular viability detection and LDH (lactate dehydrogenase) measurement

The cellular viability was detected with CCK-8 (Cell Counting Kit-8; Dojindo, Kumamoto, Japan), and LDH release was measured with a commercial kit (Roche, Basel, Switzerland), as described previously (Chen et al., 2021). Standardization of the LDH release values and cell survival rates was performed, and the results were described as percentages.

2.10. Detection of oxidative stress

The PC12 cells were harvested at 24 h after re-oxygenation to quantify the level of oxidative stress. The activities of superoxide dismutase (SOD), glutathione (GSH) and the content of malondialdehyde (MDA) were measured using commercial biochemical kits (Nanjing Jiancheng, China), according to the manufacturer’s instructions.

2.11. Quantification of ROS (reactive oxygen species) production and detection of cell apoptosis

The ROS level in PC12 cells was tested by a commercial DCFH-DA (2,7-dichlorodihydrofluorescein diacetate) kit (provided by Beyotime, Shanghai, China; #S0033), according to the manufacturer’s protocol. Apoptotic cells were detected using an Annexin V-FITC/PI Apoptosis Detection kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. Subsequently, a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was used to determine the percentage of the apoptotic cells.

2.12. Western blot analysis

The hippocampus and cortex were quickly separated on the ice plate, and the left and right sides were distinguished in 4 cryotubes and then placed into liquid nitrogen. The left hippocampus and cortex were used

| Table 1 | Primer sequence. |
|---------|------------------|
| Name    | Sequence         |
| NLRP3   | ATGCTGTTGTGACATCCTCCT |
| caspase-1 | AACAACTGGAGATGCTGCA   |
| ASC     | GTTTCAGGAGGATGCGAAA  |
| GSDMD   | GGCATAGACCTCCAGATGAGC |
| IL-1β   | GGAGGCGCTACTTCTGGA   |
| IL-18   | AACCCGCGTCGTGGCGCA   |
| GAPDH   | TGGTGGAGGAGGCTCTGAC  |

The ROS level in PC12 cells was tested by a commercial DCFH-DA (2,7-dichlorodihydrofluorescein diacetate) kit (provided by Beyotime, Shanghai, China; #S0033), according to the manufacturer’s protocol. Apoptotic cells were detected using an Annexin V-FITC/PI Apoptosis Detection kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. Subsequently, a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was used to determine the percentage of the apoptotic cells.

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Fig. 1. Chrysophanol post-treatment improved the neurological outcomes of rats suffering from CA. (A). the body weight of animals was evaluated after CA. (B). Survival rates in the Sham, CA, and CA + CHR groups. (C). Neurological scores of rats evaluated using the mNSS scoring method. (D). Representative swimming paths during the probe trial for each group. (E–G). Acquisition training in Morris Water Maze test from the 3rd to 7th day after resuscitation. (F). Path length (cm) to reach the platform; (G). Thigmotaxis (%) in Morris water; (H–K). Probe trial in Morris Water Maze test on the 7th Day after resuscitation. (H). Proportion time (%) in target quadrant; (I). Path in target quadrant (cm); (J). The number of platform crossed during 60 s after removing the platform; (K). The number of target quadrant entries during 60 s after removing the platform. Data are expressed as mean ± S.D. n = 4–6, #, P < 0.05, vs. sham group; &, P < 0.05, vs. CA group.

for Western blots; the bilateral cortex and hippocampus in the sham group were compared. The expressions of ASC, N-terminal GSDMD, cleaved-caspase-1, NLRP3, and TRAF6 in cortex and hippocampus were determined. The proteins were measured by the bicinchoninic acid (BCA) method, separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and transferred electrophoretically onto PVDF (polyvinylidene difluoride) membranes. After blocking nonspecific binding sites with BSA (bovine serum albumin, 3%), the PVDF membranes were incubated with primary antibodies: anti-caspase-1 (1:500; Proteintech, Rosemont, IL, USA), anti-NLRP3 (1:500; Cell Signaling Technology, Danvers, MA, USA), and anti-N-terminal of GSDMD (1:500; Abcam Technology, Cambridge, MA, USA), anti-ASC (1:1000; Abcam Technology, Cambridge, MA, USA), anti-TRAF6 (1:400, Abcam Technology, Cambridge, MA, USA) and anti-β-actin (1:10000; Abcam Technology, Cambridge, MA, USA), overnight at four centigrade degree, which were then incubated with a secondary antibody for one and a half hours at room temperature after TBST washing. The chemiluminescence assay was performed to identify the immunoreactive bands, and the gray value of immune reactivity was calculated using the ImageJ software.

2.13. Overexpression and knockdown of NLRP3 and TRAF6 in PC12 cells

PC12 cells were transfected with 50 nM of adenovirus (Ad-) to overexpress NLRP3 or TRAF6 (synthesized by GeneChem Co., Ltd., Shanghai, China). In addition, to knockdown NLRP3 and TRAF6, PC12 cells were treated with NLRP3 or TRAF6 siRNAs (25 nM) and their
corresponding scrambled RNAs (synthesized by GeneChem Co., Ltd.), respectively. The PC12 cells were transfected by Lipofectamine 3000 (Thermo Fisher Scientific), as per the manufacturer’s protocol. After one day, the PC12 cells were exposed to OGD/R in the in vitro model, as previously mentioned. The efficiency of overexpression and depletion was detected by Western blot assay.

2.14. Immunofluorescence staining

NLRP3 and caspase-1 expressions in the PC12 cells were determined by immunofluorescence staining. In brief, after fixed by paraformaldehyde (4%), the PC12 cells were cultured with anti-NLRP3 or cleaved caspase-1 (p20) antibody (1:200; Abcam) overnight at four centigrade degree. Subsequently, goat anti-rabbit secondary antibody (1:200; Beyotime) was incubated at twenty-five centigrade degree for 1 h. After that, 4′-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) was used to stain the nucleus of PC12 cells. Finally, protein levels were detected with a fluorescence microscope (IX81; Olympus, Tokyo, Japan).

2.15. Co-immunoprecipitation (CO-IP) assay

The samples were incubated with rabbit polyclonal IgG control antibody, anti-NLRP3 (1:200; Abcam) or anti-TRAF6 (1:200; Abcam). Next, the lysates were incubated at 4 °C overnight. Subsequently, the mixture was incubated for another 2 h after Protein A/G PLUS-Agarose
was added into the lysates. The mixture was washed and denatured using immunoprecipitation buffer, followed by immunoblotting of the eluted proteins with the anti-TRA6 or anti-NLRP3 antibody, as mentioned earlier.

2.16. Statistical analysis

The experimental data were statistically analyzed by the SPSS 25.0 software (IBM, Armonk, NY, USA), and the measurement data were expressed as mean ± SD (standard deviation). Normal distribution of the data was determined using skewness, kurtosis coefficient, and the one-sample Kolmogorov-Smirnov test. Normally distributed data were compared between groups by ANOVA (one-way analysis of variance). When the homogeneity of variance was met, the LSD (least significant difference) test was used. When the uniformity of variance was not met, the Dunnett T3 test was used. The log-rank test was used to compare survival curves, and the Kaplan-Meier survival analysis was employed for the survival analysis. Linear correlation between variables was not met, the Dunnett T3 test was used. The log-rank test was used to compare survival curves.

3. Results

3.1. General conditions of rats with or without CA/CPR

At first, the basic weight of rats in each group was similar. However, on the 3rd day after CA/CPR, the body weight of animals in the CA + CHR group was lower compared to the sham and CA groups. There was no significant difference in body weight between the sham and CA groups. On the 5th to 7th days after CA/CPR, the weight of rats in the CA and CA + CHR groups was reduced. Compared with the CA group, Chrysophanol treatment for 7 days could more significantly reduce the weight of rats (P < 0.05, Fig. 1A).

Then, the baseline physiological features of all rats, including hemodynamic parameters, temperature, and arterial blood gas tensions were recorded. Nevertheless, CA/CPR led to remarkable physiological and biochemical disorders in all the groups, except for in the sham group. Nevertheless, these changes were gradually improved from CPR to ventilation, and no alternation was found in the CA and CA + CHR groups (Table 2).

3.2. Chrysophanol post-treatment improved survival rates and prevented CA-induced neurological deficits

156 Sprague-Dawley rats were enrolled in the study, of which 45 rats were not included due to failed ventilation (n = 10), failed resuscitation (n = 13, non-ROSC), prolonged resuscitation time (n = 10, CPR more than 5 min to achieve ROSC), or death at different time points after ROSC (n = 12). Among 111 rats that were finally included in the current research, there were 30 rats in the sham group with a mortality of 0%. The survival rates on the 3rd and 7th days in the sham, CA, and CA + CHR group. Nevertheless, these changes were gradually improved from CPR to ventilation, and no alternation was found in the CA and CA + CHR groups (Table 2).

Nevertheless, compared to the sham group, the latency was longer for the CA group, Chrysophanol treatment for 7 days could more significantly reduce the weight of rats (P < 0.05, Fig. 1A).

The rats were free to swim for 1 min to find the platform after MWM test from 3 to 7 day after resuscitation (Fig. 1D). In the pre-training phase, all rats in the 3 groups swam easily. In the spatial recognition phase, the mean latency values of recognizing the platform and the length of the path to arrive at the platform gradually reduced. Nevertheless, compared to the sham group, the latency was longer for animals in the CA group (Fig. 1E; P < 0.05), the path length was greater (Fig. 1F; P < 0.05), and the proportion of thigmotaxis was higher (Fig. 1G; P < 0.05). These changes were markedly suppressed by Chrysophanol treatment (Fig. 1E-G; P < 0.05). Nevertheless, the swimming speed was not different among the three groups (data were not shown).

The mNSS scores were significantly higher in rats treated with CA/CPR surgery compared with those in sham-treated rats. Although there were no significant differences in the mNSS scores between the CA and CA + CHR groups on the first day after ROSC (P > 0.05), Chrysophanol treatment reduced the increased mNSS scores exhibited by the CA/CPR on the 3rd and 7th days after surgery (P < 0.05, Fig. 1C).

The function of spatial learning and memory was assessed using the MWM test from 3 to 7 day after resuscitation (Fig. 1D). In the pre-training phase, all rats in the 3 groups swam easily. In the spatial recognition phase, the mean latency values of recognizing the platform and the length of the path to arrive at the platform gradually reduced. Nevertheless, compared to the sham group, the latency was longer for animals in the CA group (Fig. 1E; P < 0.05), the path length was greater (Fig. 1F; P < 0.05), and the proportion of thigmotaxis was higher (Fig. 1G; P < 0.05). These changes were markedly suppressed by Chrysophanol treatment (Fig. 1E-G; P < 0.05). Nevertheless, the swimming speed was not different among the three groups (data were not shown).

The results above were suppressed by Chrysophanol treatment (Fig. 1E-G; P < 0.05). Nevertheless, the swimming speed was not different among the three groups (data were not shown).

3.3. Chrysophanol post-treatment attenuated CA-induced neuronal damage in the hippocampus

In CA/CPR-induced brain injury models, an alteration in the brain water content, which was calculated based on water content/wet weight × 100%, was found as an important indicator of brain edema. Fig. 2A illustrates that the water content in the cerebral tissues of CA-treated rats was higher compared with that in the sham-treated rats.

Table 2

| Parameters                          | Time points | CA      | CA + R  |
|------------------------------------|-------------|---------|---------|
| CPR time (s)                       | 107.9±31    | 105.5±30|         |
| Total dose of epinephrine (µg)     | 12.3±2.5    | 12.1±2.3|         |
| Mean arterial pressure (mmHg)      | 106.6±9.2   | 110.9±5.9|         |
| Rectal temperature (°C)            | 37.7±0.4    | 37.3±0.4|         |
| pH                                 | 7.4±0.05    | 7.45±0.05|         |
| PaCO₂ (mmHg)                       | 40.5±4.4    | 41.4±4.5|         |
| PO₂ (mmHg)                         | 141.7±4.2   | 145.2±4.2|         |
| mNSS                               | 134.7±3.7   | 135.3±3.5|         |
| Path length                         | 154.7±5.0   | 160±15.3|         |
| Swimming speed (cm/s)              | 148.3±2.5   | 146.8±2.5|         |
| Total dose of epinephrine (µg)     | 12.3±2.5    | 12.1±2.3|         |
| Total dose of epinephrine (µg)     | 107.9±31    | 105.5±30|         |
| Mean arterial pressure (mmHg)      | 12.3±2.5    | 12.1±2.3|         |
| Rectal temperature (°C)            | 37.7±0.4    | 37.3±0.4|         |
| pH                                 | 7.4±0.05    | 7.45±0.05|         |
| PaCO₂ (mmHg)                       | 40.5±4.4    | 41.4±4.5|         |
| PO₂ (mmHg)                         | 141.7±4.2   | 145.2±4.2|         |
| mNSS                               | 134.7±3.7   | 135.3±3.5|         |
| Path length                         | 154.7±5.0   | 160±15.3|         |
| Swimming speed (cm/s)              | 148.3±2.5   | 146.8±2.5|         |

Table 2: Physiological baseline characteristics.
Nevertheless, Chrysophanol post-treatment dramatically reduced the brain water content on the postoperative 7th day.

The results of hematoxylin and eosin (HE) staining exhibited absence of damaged cells and normal morphology in hippocampal CA1 in sham-operated rats. However, a great number of damaged cells with obvious neuron loss were observed in the same region in the CA-treated rats. Additionally, the morphology was normal with rare damaged cells in the same region after Chrysophanol post-treatment (Fig. 2B).

The number of hippocampal neuronal cells was counted on the 7th day after recovery from CA/CPR in all the groups. Several atrophic neurons with damaged nuclei and shrunk cytoplasm were found in the animals of CA group, while the morphology remained unchanged in the sham group, according to the results of the Nissl staining. Besides, the neuronal loss was prevented after Chrysophanol post-treatment, compared to the CA group (Fig. 2C and D, P < 0.05).

In the present study, the number of TUNEL-positive cells in the CA1 region was significantly elevated following CA and resuscitation. However, neurons in the CA1 region displayed a regular arrangement in the sham-operated rats. Similarly, Chrysophanol post-treatment remarkably decreased the number of CA-induced TUNEL-positive cells in the CA1 region of the hippocampus at 7 days after CA/CPR (Fig. 2E and F, P < 0.05).

3.4. Chrysophanol post-treatment alleviated CA-induced neuronal pyroptosis possibly in parallel with suppressed TRAF6 expression

It has been demonstrated that pyroptosis might play a crucial role in CIRI. Subsequently, we attempted to indicate whether Chrysophanol-mediated neuroprotection is involved in suppressing CA-induced pyroptosis in vivo, in which the pyroptosis-related proteins were analyzed. We firstly investigated the effect of Chrysophanol on the assembly of ACS, NLRP3 inflammasomes, cleaved caspase-1, and GSDMD-N (N-terminal GSDMD). Western blotting and RT-qPCR clearly indicated that CA increased NLRP3, ASC, cleaved caspase-1, and GSDMD-N levels in both cortex and hippocampus at 7 days after CA. Chrysophanol post-treatment also significantly inhibited the mRNA and protein levels of
NLRP3 and ASC. Moreover, Chrysophanol post-treatment markedly suppressed the activity of cleaved caspase-1. In addition, we further found similar results, in which Chrysophanol post-treatment reduced the increased level of GSDMD-N after surgery (Fig. 3 A–F, \( P < 0.05 \)).

Pyroptosis, a newly discovered inflammation-mediated programmed cell death, amplified tissue injury or induced cell death by triggering excessive inflammatory damage. On the 7th day after CA, we determined whether Chrysophanol post-treatment can exert a neuroprotective effect by suppressing pyroptosis-related inflammatory factors using ELSIA. The pyroptosis-related inflammatory products (IL-1\( \beta \) and IL-18) were also remarkably abated after Chrysophanol post-treatment (Fig. 3 G, \( P < 0.05 \)). Furthermore, the expression level of TRAF6 was significantly upregulated after CA, while it was markedly downregulated after Chrysophanol post-treatment (Fig. 3 H and I, \( P < 0.05 \)).

Taken together, these findings suggested that Chrysophanol post-treatment substantially restrained formation of NLRP3 inflammasomes and cleavage of caspase-1 and GSDMD-N, thereby preventing the maturation of IL-1\( \beta \) and IL-18 in the brain after CA in parallel with inhibition of TRAF6 expression.

3.5. Chrysophanol post-treatment protected PC12 cells against OGD/R-induced pyroptosis

One of the primary pharmacological functions of Chrysophanol, an important component of Rhubarb, is associated with its anti-inflammatory properties. Firstly, we used different concentrations of Chrysophanol to investigate its neuroprotective role. Chrysophanol alone had no significant influence on cellular viability and LDH release (data are not shown). As demonstrated in Fig. 4 A and B, 50 \( \mu \)M Chrysophanol did not improve the cellular viability as evidenced by CCK-8 assay and LDH release. However, Chrysophanol post-treatment elicited a neuroprotective effect under OGD/R conditions in dose-dependent manner. Since the most appropriate concentration was 100 \( \mu \)M, it was selected for subsequent experiments (\( P < 0.05 \)).

In order to further testify that pyroptosis is the major form of programmed cell death in OGD/R-stimulated injury, we used MCC950, a specific inhibitor of NLRP3, and necrosulfonamide (NSA), a chemical inhibitor of gasdermin D, to inhibit pyroptosis. Compared with OGD/R or OGD/R + Vechile group, both MCC950 and NSA significantly suppressed neuronal death, as evidenced by improved cellular viability and decreased LDH release in the absence of Chrysophanol (Fig. 4C–F, \( P < 0.05 \)).
However, cellular viability and LDH release were not significantly different between MCC950 and MCC950 + CHR groups or between NSA and NSA + CHR groups, indicating that no additional effect was shown by MCC950 or NAS in Chrysophanol-treated PC12 cells.

Next, we verified that Chrysophanol alone or combined with MCC950 or NAS had protective effect against OGD/R-caused pyroptosis by detecting the levels of pyroptosis-related proteins. Western blotting revealed that the levels of NLRP3, cleaved caspase-1 and GSDMD-N were all inhibited in Chrysophanol, MCC950 or CHR + MCC950 group under OGD/R condition (Fig. 4G and H, P < 0.05). Similarly, the level of GSDMD-N was markedly suppressed in the Chrysophanol, NSA and CHR + NSA groups (Fig. 4I and J, P < 0.05). Moreover, the levels of pyroptosis-associated inflammatory factors (IL-1β and IL-18) were also reduced after Chrysophanol alone or combined with MCC950 or NAS treatment, as measured by ELISA (Fig. 4K, P < 0.05). However, there was no significant difference in pyroptosis-related indicators between
CHR and MCC950 + CHR groups or between CHR and CHR + NSA groups.

To get more evidence, NLRP3 was overexpressed or knocked down in OGD/R exposed-PC12 cells. Overexpression of NLRP3 enhanced the expression of pyroptosis-related proteins and inflammatory products in the OGD/R-stimulated PC12 cells. Although silencing of NLRP3 by using siRNA (small interfering RNA) did not show additional protective effects for Chrysophanol-treated cells, the neuroprotective effects of Chrysophanol post-treatment were neutralized in NLRP3 overexpressed PC12 cells under OGD/R (Fig. 4L–N, *P < 0.05). Based on these results, we confirmed the important role of NLRP3-regulated pyroptosis in Chrysophanol-mediated CIRI.

Collectively, the above-mentioned findings suggested that NLRP3-mediated pyroptosis was the major apoptotic signaling pathway modulated by Chrysophanol post-treatment in the setting of OGD/R-induced injuries in PC12 cells.

3.6. Chrysophanol post-treatment inhibited pyroptotic cell death by inhibiting the interaction between TRAF6 and NLRP3

TRAF6 was determined in vitro, and the results showed that the expression level of TRAF6 was upregulated during OGD/R-triggered damage, while it was downregulated after Chrysophanol post-treatment (Fig. 5A and B, *P < 0.05). Additionally, the results of the Pearson’s correlation analysis indicated that the levels of TRAF6 and GSDMD-N showed positive correlation (Fig. 5C and D, *P < 0.05).

To confirm that Chrysophanol post-treatment is protective against OGD/R-induced injury in a TRAF6-dependent manner, we also performed gain- and loss-of-function analyses of TRAF6 in PC12 cells. Firstly, knockdown of TRAF6 appeared protective against OGD/R-induced neuronal death, while overexpression of TRAF6 exhibited an opposite effect (Fig. 5E and F, *P < 0.05). Previous studies had clearly demonstrated that TRAF6 plays indispensable roles in regulating oxidative stress in CIRI injury. Therefore, to get more solid evidence, we further examined whether TRAF6 overexpression or knockdown influenced oxidative stress in OGD/R-treated PC12 cells in the absence or presence of Chrysophanol. Firstly, the content of MDA in OGD/R-operated PC12 cells was decreased after Chrysophanol post-treatment. Contrarily, protective factors, including GSH and SOD, were all increased. Similarly, overexpression of TRAF6 suppressed the effect of Chrysophanol on oxidative stress induced by OGD/R exposure, while, TRAF6 consumption played additional protective roles in oxidative stress of Chrysophanol-treated PC12 cells (Fig. 5G–I, *P < 0.05).

Moreover, overexpression of TRAF6 also reversed the neuroprotective effects of Chrysophanol post-treatment, because OGD/R-re-
demonstrated that post-operative interventions of inhalational anesthetics (Hwang et al., 2017), adenosine agonist (Grewal et al., 2019), and other agents (Yang et al., 2018) were used as pharmacological mitigants to prevent lethal CIRI, which could be ideal alternatives that might substitute preconditioning. Chrysophanol plays an essential role in CIRI. A growing body of evidence demonstrated that Chrysophanol promoted neuronal survival in the brain after CIRI because of its strong anti-oxidative stress, anti-apoptosis and anti-inflammation properties (Zhao et al., 2018b; Zhao et al., 2016; Zhang et al., 2014). Thus, the most remarkable finding of the present study was that Chrysophanol, an important component of Rhubarb, exerted robust protective effects against CIRI, as evidenced by improved survival rates, neurological outcomes, and decreased brain damage. Moreover, we further confirmed that Chrysophanol post-treatment also protected PC12 cells against OGD/R-induced injury, as documented by increased cellular viability and alleviated LDH release. Therefore, Chrysophanol post-treatment showed a neuroprotective effect, and it was a promising therapeutic option for CIRI.

During CIRI, necrotic neuronal cells may release various DAMPs (damage-associated molecular patterns), which serve as inflammatory substances to initiate inflammatory cascade reactions. Pyroptosis, identified as an inherently inflammatory programmed cell death, has been found to be associated with the development and progression of post-CA brain injury (Jiang et al., 2020; Chang et al., 2020). Several experimental studies have reported that the pyroptosis-relevant molecules are highly expressed in the ischemic brain, indicating that pyroptosis is critical for CIRI (Liang et al., 2020; Xia et al., 2018). Thus, pyroptosis and its relationship with inflammation have noticeably
attracted clinicians’ attention for the treatment of CIRI. Distinct from other forms of cell death, pyroptosis has its distinctive morphological features and molecular mechanisms. As a crucial pyroptosis-associated mediator, NLRP3 (NOD-like receptor family pyrin domain containing 3) inflammasomes generally contain NLRP3, apoptosis-associated speck-like proteins (e.g., ASC), and pro-caspase-1. Recent studies have demonstrated that genetic deficiency of NLRP3 inflammasomes efficaciously relieved ischemic brain injury-mediated pyroptosis (Yang et al., 2014). Consistent with previous studies, we found that NLRP3 inflammasome pathway was activated by CA and OGD/R, leading to an excessive inflammatory cascade response. Correspondingly, Chrysophanol post-treatment strongly suppressed CIRI-induced NLRP3 inflammasomes, suggesting that the neuroprotective effects of Chrysophanol post-treatment might be related to its anti-pyroptotic features. In addition, the mature NLRP3 inflammasomes complex could also cleave pro-caspase-1 into active caspase-1, which was recognized to modulate pyroptotic cell death. According to the previous studies, the expression levels of caspase-1 were increased in ischemic stroke models. Moreover, caspase-1 inhibition (Liang et al., 2021) or knockout (Schielke et al., 1998) exhibited a neuroprotective effect against CIRI. Consequently, active caspase-1 triggered maturation and secretion of IL-18 and IL-1β. We, in the present study, found that Chrysophanol post-treatment significantly suppressed the secretion of IL-18 and IL-1β and the expression level of cleaved caspase-1 during CIRI. Additionally, active caspase-1 subsequently regulated the release of GSDMD-N, which specifically interacted with phospholipid molecules in the plasma membrane and then caused formation of pores, eventually resulting in pyroptosis (Wang et al., 2020). Thus, GSDMD was featured by a pivotal executioner of caspase-1-dependent pyroptosis. Similarly, Chrysophanol post-treatment markedly neutralized the increased level of GSDMD-N after CA and OGD/R. To achieve more solid evidence, the specific inhibitors of NLRP3 and GSDMD, MCC950 and NSA were added to rescue PC12 cells from OGD/R, indicating that pyroptosis is the main cell death in CIRI. More importantly, overexpression of NLRP3 not only exacerbated OGD/R-induced cell injury, but also reversed the effects of Chrysophanol post-treatment on the pyroptosis-related indicators, while knockdown of NLRP3 showed opposite effects. Based on these findings, it was demonstrated that Chrysophanol post-treatment could exert robust protective effects through suppressing pyroptosis.

To date, animal experiments have also indicated that Chrysophanol relieves CIRI-induced brain damage through attenuation of neuroinflammation (Zhao et al., 2016; Zhang et al., 2014). Nevertheless, few studies have reported the anti-pyroptotic effects of Chrysophanol on post-CA brain injury. According to the findings of the present study,
Fig. 5. (continued).
overexpression of NLRP3 almost completely suppressed the protective effects of Chrysophanol, suggesting that pyroptosis was identified as a major neuronal death form modulated by Chrysophanol post-treatment during CIRI. Therefore, further delineation about the underlying molecular mechanisms of anti-pyroptotic effects of Chrysophanol is of great significance to develop novel therapeutic strategies.

TRAF, which is composed of 7 members (TRAF1–7), is an important multifunctional protein (Lee and Lee, 2002). TRAF6, distributed widely and abundantly in the brain, is the most important member of TRAF family, because of the smallest homology and the greatest difference in C-terminal domain. Studies have increasingly found that TRAF6 inhibition significantly restrained the degree of reactive oxygen species (ROS)-related oxidative stress (Yan et al., 2020; Peng et al., 2020) and NF-κB-mediated inflammation in ischemic injury (Chen et al., 2019). Mechanistically, TRAF6 activation, resulting in the elevated ROS production, may contribute to the presence of pyroptosis. Besides, TRAF6 was dramatically upregulated in the peripheral blood of ischemic stroke individuals (Wu et al., 2013) and TRAF6 gene polymorphisms were related to the increased susceptibility of ischemic stroke in Southern Chinese Han population (Su et al., 2015). Thus, TRAF6 could be defined as a bridge between oxidative stress and neuroinflammation. Oxidative stress occurs during CIRI due to an imbalance between pro-oxidant and anti-oxidant activities, resulting in surplus formation of ROS, also recognized as a causative event of neuroinflammation and one important DAMP, which could initiate a series of cellular signaling cascades that eventually trigger pyroptosis (Yan et al., 2020; Xia et al., 2018). Our results also showed that Chrysophanol post-treatment suppressed oxidative stress in OGD/R-treated PC12 cells. Additionally, TRAF6-deletion cells were more resistant to OGD/R-triggered oxidative stress, as shown by markedly reserved levels of SOD and GSH, as well as

**Fig. 6.** Chrysophanol post-treatment protected against OGD/R-induced pyroptosis through suppressing TRAF6-NLRP3 interaction. (A–B). Representative blots and quantitative analysis showed that TRAF6 overexpression or knockdown altered the expression of pyroptosis-related proteins with or without Chrysophanol treatment under OGD/R condition. (C). The level of IL-1β and IL-18 in indicated groups, quantified by ELISA. (D). Representative confocal microscopic images showed co-localization of NLRP3 (red) with active caspase-1 (green), adenovirus (Ad-) to overexpress TRAF6 and si-TRAF6 was used to reveal the relationship between CHR and pyroptosis. (E–F). The co-localization of TRAF6 and NLRP3 was indicated by Co-Immunoprecipitation assay. n = 3, experiments, #, P < 0.05, Data are expressed as mean ± S.D.
Fig. 6. (continued).
reduced ROS production and MDA content. In contrast, TRAF6 over-expression made the PC12 cells more vulnerable to OGD/R-induced oxidative stress. In addition, gain- and loss-of-function analyses in vitro also unraveled that TRAF6 promoted the progression of OGD/R injury by enhancing NLRP3-related pyroptosis. Furthermore, the expression levels of TRAF6 and GSDMD-N showed positive correlation with each other in OGD/R-stimulated PC12 cells. We further confirmed that TRAF6 ablation markedly suppressed pyroptotic signaling pathway. Similarly, overexpression of TRAF6 deteriorated pyroptotic cell death, and suppressed the protective effects of Chrysophanol post-treatment. More importantly, unlike silencing NLRP3, Chrysophanol post-treatment could provide additional neuroprotection in si-TRAF6-treated cells. Therefore, our results indicated that TRAF6 functioned as an important promoter of pyroptotic cell death, which was regulated by Chrysophanol. However, the detailed intracellular molecular mechanism is still unclear. A previous study demonstrated that TRAF6 induced ROS overproduction and neuroinflammation through directly interacting with Ras-related C3 botulinum toxin substrate 1 (Rac1) (Li et al., 2017). Additionally, the anti-inflammatory effect of DJ-1 was involved in dissociation of NLRX1 from TRAF6 during CIRI (Peng et al., 2017). Additionally, the anti-inflammatory effect of DJ-1 was involved in dissociation of NLRX1 from TRAF6 during CIRI (Peng et al., 2020). According to these findings, we found that TRAF6 interacted with NLRP3 to trigger pyroptotic inflammatory process. In agreement with previous findings mentioned above, Chrysophanol post-treatment significantly attenuated the interaction between TRAF6 and NLRP3, suggesting that Chrysophanol has a prospective anti-pyroptosis feature, and the underlying mechanism is involved in regulation of the TRAF6-NLRP3 axis. However, some limitations of the present study should be addressed. First, we do not in fact detect the expected concentrations of Chrysophanol in the circulation and in the target tissue of brain. Secondly, Chrysophanol alleviated pathologies of CIRI deserves to be further explored in the clinical practices.

In summary, TRAF6 was induced following CIRI and promoted ROS-mediated oxidative stress, which in turn enhanced pyroptosis. Besides, TRAF6 also directly interacted with NLRP3 and potentiated the activation of NLRP3 inflammasomes and the latter ultimately contributed to pyroptotic cell death. Hence, our present study had revealed that Chrysophanol, a natural anthraquinone isolated from Rubarb, may potentially attenuate CIRI through the TRAF6-dependent inactivation of NLRP3-related pyroptosis, providing an alternative option for treating CA-induced brain injury.

Authors’ contributions

Zhi Ye and Pingping Xia conceived and designed the experiments. Murat Marjan, Zhouyi Liu, Wanzhou Qiu and Pingping Xia performed the experiments. Qian Zhang, Chen Cheng and Yuanyuan Tao analyzed the data. Jie Minxi Zhao, Zhihui Wang, Yuanyuan Tao and Chen Cheng provided reagents/materials/analysis tools. Pingping Xia, and Murat Marjan wrote the manuscript. Zhi Ye Conceptualization, Methodology, Funding acquisition, Project administration, Supervision.

Declaration of Competing Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, or publication of this article.

Data availability

No data was used for the research described in the article.

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