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ARTICLE

Therapeutic effect of *Polygala crotalarioides* on a middle cerebral artery occlusion/reperfusion (MCAO) model

Yili Zhang[1], Qinghua Chen[2], Yonghua Li[3], Yongsheng He[4*]

1 Cytocraft Biopharmaceutical Co.,Ltd

2 Yunnan University of Traditional Chinese Medicine, Yunnan key laboratory of Dai Medicine and Yi Medicine

3 Yunnan Yihua Agricultural Biological Some Company, Yunnan, Kunming 650500

4 Yunnan Labreal Biotechnology Co., Ltd, Yunnan, Kunming, 650000

Corresponding Author: lab_hys@163.com

Abstract

Background:Ischemic stroke is a devastating disease with high mortality and disability, causing a significant economic and social burden, and severe brain injury. Polygala crotalarioides has shown medical potential as a highly-effective, hormone-free, natural anti-fatigue and cardiotonic agent, among the Wa ethnicity. Previous studies have shown that Polygala crotalarioides (*P. crotalarioides*) contains many active ingredients, but the pharmacological mechanism of it in MCAO/R is not investigated.

Methods: The in vivo MCAO/R rat model was constructed. Modified Neurological Severity Scores (mNSS) and 2,3,5-triphenyltetrazolium chloride staining (TTC) were performed to reveal the protective role of *P. crotalarioides* after MCAO/R. The apoptosis related proteins in the hippocampus region were detected by western-blot assay. In order to reveal the proliferation of neurons and Nissl body, immunofluorescence (IF) assays and Nissl staining were performed in vitro, respectively.

Results: Our results showed that *P. crotalarioides* treatment significantly suppressed neuronal apoptosis. Western-blot analysis indicated that Bax/Bcl-2 and cleaved-caspase-3 were decreased after *P. crotalarioides* treatment. The score of mNSS and the infract area in *P. crotalarioides* group were alleviated compared with the MCAO/R group. The results of Nissl staining suggested that Nissl body was enhanced under *P. crotalarioides* treatment. And the proliferation of neurons was positively regulated by *P. crotalarioides.*

Conclusions: The present study suggested that *P. crotalarioides* exhibited neuroprotective effect that in MCAO/R, possibly through reverse the neuron apoptosis and proliferation.

Introduction

Stroke is primarily caused by hemorrhage and ischemia, of which ischemia stroke accounts for 85%, ischemic stroke is one of the leading causes of neurological disability and death in the world[1]. Stroke causes a range of injuries including post-ischemic neuroinflammation and cerebral edema. Reperfusion

treatment including intravenous thrombolysis, arterial thrombolysis and thrombectomy are effective treatment methods of rescuing the remaining neurons in patient after ischemia stroke[2]. However, the clinical applications have a narrow therapeutic window and treatment beyond the therapeutic window can exacerbate brain damage[3], the reperfusion processes may further aggravate the initial ischemic injury after ischemic attack, and this process is termed cerebral ischemia/reperfusion (I/R) injury[4]. Thus, it is urgent to uncover the mechanisms of ischemic stroke and neuron regeneration which can be useful in the development of new treatment methods. Possible mechanisms of ischemic stroke include apoptosis, coxidative stress and energy metabolism disorders[5]. Apoptosis is an energy-dependent, programmed form of cell death, and the morphological and biochemical evidences of apoptosis have been assessed in experimental animal models of ischemic brain injury[6].

The natural products have structure diversity and bioactivity diversity and can serve as potential resource for novel drugs. Recent studies have identified a range of natural products with the potential for stroke treatment. Geraniin exerts neuroprotective effects against brain I/R injury and associated with activation of the NRF2/HO-1 pathway[4]. The traditional Chinese Medicine Naoxintong exhibits anti-cerebral ischemia pharmacological effect by reducing the DNA methylation level of NogoA pathway after ischemic stroke and further inhibits the expression of NogoA/RhoA/ROCK pathway[7]. There are about 500 species of plants (Polygala) in Polygalaceae, distributed in temperate and subtropical regions of Eurasia and America, and there are 42 species and 8 varieties in China, mainly distributed in Yunnan, Guizhou, Guangxi and Guangdong[8]. Polygala. crotalarioides (*P. crotalarioides*) Buch. Ham. produced in northern Yunnan, southern Sichuan and southern Xizang, the whole plant, especially the root has the effect of activating blood circulation to dissipate blood stasis and calming the mind [9]. The plant is also known as "YaMoNiang", meaning "red herbal medicine" in southern Yunnan province where Wa ethnicity live in. It is a precious Chinese herbal medicine spread in the Wa ethnicity of Yunnan. In the Wa ethnicity, *P. crotalarioides* as a natural strong agent without hormones to combats fatigue and tonic the heart. In our previous investigation, three categories of components were isolated from the *P. crotalarioides*, including oligosaccharide polyesters, triterpenoid saponins, xanthones and their derivatives, among which sugar esters are the most abundant class of compounds. From the pharmacological perspective, some xanthones show the inhibitory activitiy against Xanthine Oxidase[10], while triterpenoid saponin notoginsenoside R1 exhibits neuroprotective activity[11]. The biological activity study of *P. crotalarioides* is still incomplete and deserves our further investigation.

In our study, we constructed a rat model of MCAO/R to value the protective effect of *P. crotalarioides* and investigated the preliminary mechanism of action. We indicated that *P. crotalarioides* attenuated MCAO/R induced neuronal apoptosis in vivo and hence promoted neural regeneration.

Materials and methods

Middle cerebral artery occlusion-reperfusion (MCAO/R) Model establishment

This laboratory animal ethics (KMMU2020502, 20200815) has been approved by the Kunming Medical University ethics committee. The experimental procedures strictly followed the guidelines for the management and the usage of laboratory animals formulated by the national institutes of health according to the previous studies [12, 13].

All rats were divided into 4 groups: sham group (n=6), MCAO/R group (n=6), MCAO/R + *P. crotalarioides* group (n=6) and MCAO/R group + Naoxintong (n=6). In brief, rats were anesthetized by injection of sodium pentobarbital (42 mg/kg), and they were fixed on the stereotaxic apparatus after they have become completely unconscious. After hair removed, an incision was made along with midline cervical to exposed the right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA). CCA and ECA were tied off and ICA was closed. The monofilament suture was inserted from ECA and advanced into ICA. 2 hours after surgery, 24 hours reperfusion was performed by removing the monofilament suture. Sham rats were subjected to the same procedure without MCAO/R.

A stock solution of *P. crotalarioides* (Yun County, Yunnan Province, China) was prepared in 5M NaOH, Ph 7.4 (adjusted with 1M HCl), and further diluted in 0.9% normal saline for in vivo studies. MCAO/R + *P. crotalarioides* group and MCAO/R group + Naoxintong group were injected with *P. crotalarioides* (200 mg/kg) and Naoxintong (110 mg/kg)[14] intraperitoneally immediately after reperfusion and continuously injected for 14 days after ischemic stroke, respectively. The MCAO/R group rats were injected without any treatment.

Neurological function test

Neurological function was assessed on modified Neurological Severity Scores (mNSS) [15] on post-MCAO/R

days 1, 3, 7, 14 and 21 days. The mNSS is a composite test of motor, sensory systems, reflexes and balance, the scoring method follows the description[15]. The higher scores indicate more severe neurological impairment of CNS.

2,3,5-triphenyltetrazolium chloride (TTC) staining

2,3,5-triphenyltetrazolium chloride (TTC, Sigma China) staining was use to evaluate the infarct volume[16], rats were euthanized immediately after reperfusion model establishment, then brain was harvested and frozen for 5 minutes at -80 ℃. 2 mm brain slices were cut and then immersed in 2% TTC solution for 30 minutes at 37 ℃ in dark. The stained brain slices were labelled and photographed with a digital camera (Canon IXUS175; Canon, Inc.), and the infarct volume was calculated using Image-Pro Plus image processing software (infarct volume = infarct area of each slice × 2 mm).

Western blotting assay

Rats were euthanized followed the guideline of Kunming medical university, and the brain tissue was extracted. RIPA lysate (Beyotime Institute of Biotechnology) was used to extract total protein. Then, the protein separation was performed by SDS-PAGE and transferred onto PVDF membrane. 5% BSA to PVDF membrane for blocking, and membrane incubated at room temperature for 1 h. The PVDF membrane was incubated overnight with diluted rabbit monoclonal antibody at 4°C. Goat Anti-Rabbit IgG H&L (HRP, 1:10000, ab7090) was added at room temperature for incubation in next day. ECL reagent (Beyotime) was used for development, and observed and taken pictures in ChemiDoc XRS+ gel imaging system (Bio-Rad, Hercules, CA, USA). β-actin was internal reference, and the average relative protein expression was expressed as the grayscale value of the target protein relative to β-actin, which was analyzed using Image J software (National Institutes of Health, USA).

Enzyme linked immunosorbent assay (ELISA)

The secretion of pro-inflammatory cytokines IL -1β and IL-18 in brain tissue were detected by ELISAs kits (R&D System, Minneapolis, MN, USA). The experimental operation was strictly performed accordance with the manufacturer.

Nissl staining

Brain samples were collected, paraffin-embedded, and sectioned at 4-μm coronally. Coronal slices thick were set for Nissl staining[17]. The experiment was performed according to the instruction of Nissl staining kit (Solarbio, China). After dehydration with alcohol and soaked in xylene, brain slices were stained with thionine. Then, obtained the image under bright field microscope (Leica, Germany).

Immunofluorescence staining[18]

Brains were sliced into 10 μm thick coronal sections using a cryostat, then, the sectioned tissues were placed into individual slides and dried at 37℃ containers for 72 h before immunofluorescene staining. After the slides were washed with phosphate-buffered saline (PBS) and permeabilized with 0.3% triton X-100 for 30min, blocked by 10% goat serum for 60 min, then incubated with primary antibodies 5-bromo-2'-dexoyuridine (BrdU) (1:250, ab6326, abcam), Neuronal nuclei (NeuN) (1:1000, ab104224, abcam) at 4 ℃ overnight. After that, the cells were incubated with secondary antibody including Fluorescein at room temperature for 2 hours in dark. All nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 2 minutes in dark. Image was performed on a fluorescence microscope.

Statistical analyses

All data were analyzed by GraphPad Prism 9.0 and expressed as mean ± standard deviation (SD). Student's t-test were used for differences between two groups, differences among mutiple groups were analyzed by one-way ANOVA with a Tukey test. All comparisons for which $p<0.05$ were considered a significantly differences.

Results

***P. crotalarioides* treatment ameliorated reduced neurological scores and infract volumes under MCAO/R**

To test the neuroprotective effects of *P. crotalarioides*, neurological score and 2,3,5-Triphe-nyltetrazolium chloride (TTC) staining were performed on middle cerebral artery occlusion method (MCAO) rats. TTC staining was used to value the infarct area. As shown in fig. 1A and 1B, the infract volume of MCAO/R rats was significantly increased compared to sham group. while P. crotalarioides and pocitive control reversed the infract volumes caused by ischemia. Also, the score of the mNSS was significantly increased in MCAO/R group compared with sham group (fig. 1C), indicating the obverse neuron injury caused by ischamia. While *P. crotalarioides* and naoxintong treatment significantly decrease the score compared with MCAO/R group. These results showed that *P. crotalarioides* treatment reversed the neurological deficit caused by MCAO/R.

***P. crotalarioides* normalized the expression of apoptosis-related proteins and the secretion of inflammatory cytokines in post-stroke mouse**

To prove the *P. crotalarioides* reduced apoptosis of hippocampus neurons, we isolated the hippocampus of mice and performed Western-blot analysis to detect the apoptosis related proteins Bax, cleaved caspase3, and Bcl-2. As shown in fig. 2A-C, the ratio of Bax and Bcl-2 was increased after

ischemia, while the ratio was decreased after P. crotalarioides and naoxingtong administration. Same trend was observed in the expression of cleaved caspase3.Also, the results of ELISA showed that the secretion of IL-1β and IL-18 were increased in MCAO/R model compared to sham group, while which alleviated after *P. crotalarioides* and naoxingtong administration (fig. 2D-E). The results suggested that *P. crotalarioides* played a neuroprotective role by suppressing the apoptosis of hippocampus neurons and decreasing the neuron inflammation.

***P. crotalarioides* promoted the proliferation of neurons in vivo**

To evaluate the proliferation of neurons, the expression of Brdu and NeuN were detected by immunofluorescence. Neuronal nuclear antigen (NeuN) is a marker of mature neuronal cells [19] and Brdu labels the newborn cells[20]. The Representative fluorescent images of brain from different groups rats are shown in fig. 3A-3C. MCAO/R rats showed extensive neuronal loss, in contrast, *P. crotalarioides* and Naoxingtong treatment significantly increased the positive rate of NeuN and Brdu. These results indicated that *P. crotalarioides* increased proliferation of neurons.

***P. crotalarioides* alleviated the neuropathic damage under MCAO/R**

We used Nissl staining to observe the role of *P. crotalarioides* on neuropathic damage caused by ischemia. In MCAO/R group, Nissl body decreased in hippocampus, and karyopyknosis and dark staining was observed in neuron nuclei (fig. 4). In contrast, the number of Nissl bodies were increased and the morphological changes were alleviated in the *P. crotalarioides* group and positive control group compared to the MCAO/R group. The results suggested that *P. crotalarioides* alleviated the neuropathic damage after MCAO/R.

Discussion

Ischemic stroke is a leading cause of death and disability globally and a long-term post-storke care is also required[21]. Although there are many mechanisms of stroke pathogenesis, mounting evidence suggests that ischemic injury and inflammation are important mechanisms. The sudden occlusion of blood vessels due to thrombosis or embolism cause the loss of oxygen and glucose in the brain tissue[22]. Reperfusion of ischemic brain tissue is essential to restore normal function but the I/R damage it causes is also fatal. Intravenous recombinant tissue plasminogen activator (rt-PA) is the drug approved by US, which was used in three hours after symptom onset[23], but only 3.4-5.2 % of patients received this treatment in 2009[24], this can be partly attributed to the delivery difficultied of therapeutics across the BBB[25]. Recently, stem cell therapy following stroke can improve cognitive dysfunction and functional recovery by regulating angiogenesis and neurogenesis, but the long term impacts of the therapy is lacking [26]. Our results showed the MCAO/R modelling induced remarkable increase in the cerebral infract volume, *P. crotalarioides* reversed the infract volumes caused by ischemia.

Up to now, naturals products have been widely investigated as potential prodrugs. Astragalus saponins are one of the main active components of traditional Chinese herbal Astragali Rsdix, which protect experimental stroke by enhancing proliferation of neural stem cells through targeting Akt[27]. Tenuifolin has potential benefits for the study of learning and memory deficits in APP/PS1 transgenic AD mice and it may inhibit the mitochondrial membrane-potential loss and then alleviate the apoptosis of neurons, it is isolated from the Polygala tenuifolia [28]. Polygalaceae family which includes about 22 genera and 1300 species, *P. crotalarioides* is one of the species in polygalaceae family[10]. At present, researchers have isolated differnet compounds such as oligosaccharide polyesters, triterpenoid saponins and xanthones from P. *crotalarioides*[29], these natural products have diverse biological activities. Senegins I and II are triterpenoid saponins extracted from the Polygala senega var. latifolia, which significantly reduced the blood glucose level of KK-Ay mice[30]. The activity mechanism study of MCAO is lacking, so we tested the activity of *P. crotalarioides* in a MCAO/R model. The TTC staining and Nissl staining results suggested that *P. crotalarioides* inhibits cerebral damage following MCAO/R, and the IF results showed *P. crotalarioides* treatment increased proliferation of neurons.

Apoptosis includes extrinsic and intrinsic pathways, the initiation of these two pathways will recruit downstream apoptotic molecules such as Bax (pro-apoptotic member), Bcl-2 (anti-apoptotic member), and activated Caspase-3 to execute the cell death cascade[31]. Bax, Bcl-2 and Caspase-3 are key molecules in the process of cell apoptosis, Bcl-2 protein inhibits cell apoptosis, and Bax protein combines with Bcl-2 to form a complex, which promotes the degradation of Bcl-2 to relieve the inhibitory effect of Bcl-2 on cell apoptosis[32]. Our results revealed that *P. crotalarioides* was able to reduce nerve damage in MCAO/R mice by inhibiting the apoptosis of neurons via regulating the expression of Bax/Bcl2 and Caspase-3. And the *P. crotalarioides* may be a potential bioactive molecule for MCAO/R.

Conclusion

The study demonstrated that P. crotalarioides could improve neurological function, alleviate the neuropathic damage and inhibit neuroinflammation under MCAO/R. The mechanism of the effects may be related to supressed neuronals apoptosis. The results provided for further analysis the potential role of e P. crotalarioides as a therapeutic agent for MCAO/R.

Availability of data and materias

All data and materials are available from the corresponding author on reasonable request.

Funding

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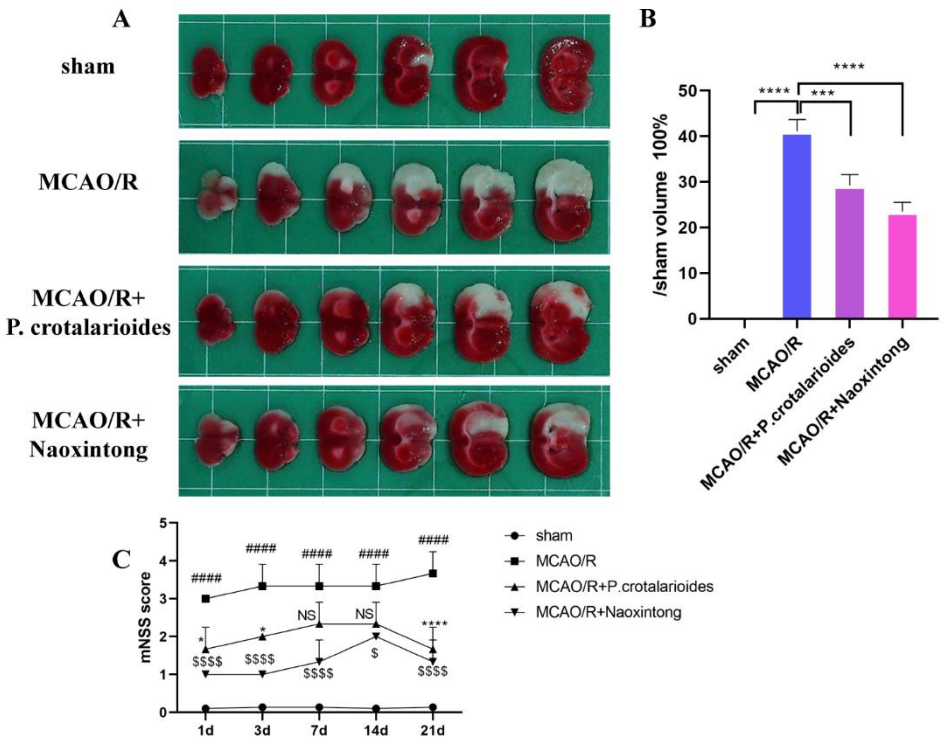

Fig.1. P. crotalarioides treatment ameliorated reduced neurological scores and infract volumes under MCAO/R. A:Representative TTC staining and (B) quatification of the infract volume at 3 days after MCAO/R. n=6 for each group. C: mNSS score on 1,3,7,14,21 days after MCAO/R; (Error bars represent mean ± SD, Magnification: 400×; sham vs MCAO/R: #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001; MCAO/R vs MCAO/R + P. crotalarioides: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; MCAO/R vs MCAO/R + Naoxintong: $p < 0.05, $$p < 0.01, $$$p < 0.001, $$$$p < 0.0001.)

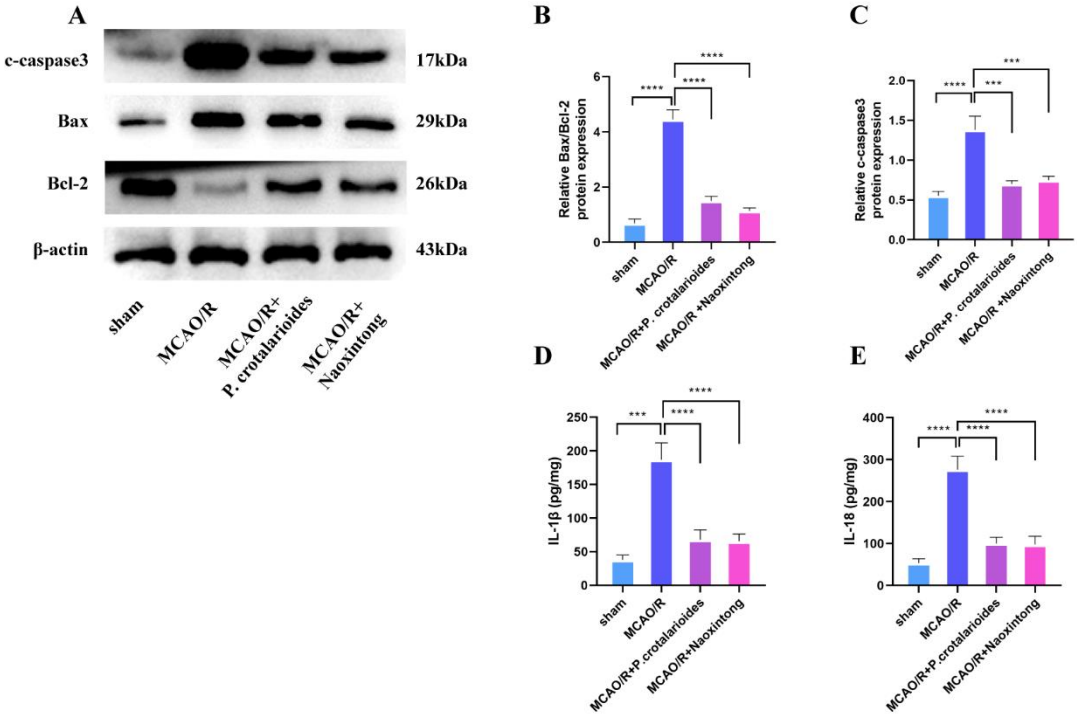

Fig.2. P. crotalarioides normalized the expression of apoptosis-related proteins and the secretion of inflammatory cytokines in post-stroke mouse. A: Western blot assay of apoptosis-related proteins in nuuron. B: Quantification of Bax, Bcl2 and cleaved-caspase3 western blot band intensity. C: ELISA analysis of the secresion of IL-1β and IL-18 in neuron. (Error bars represent mean ± SD, Magnification： 400×; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.)

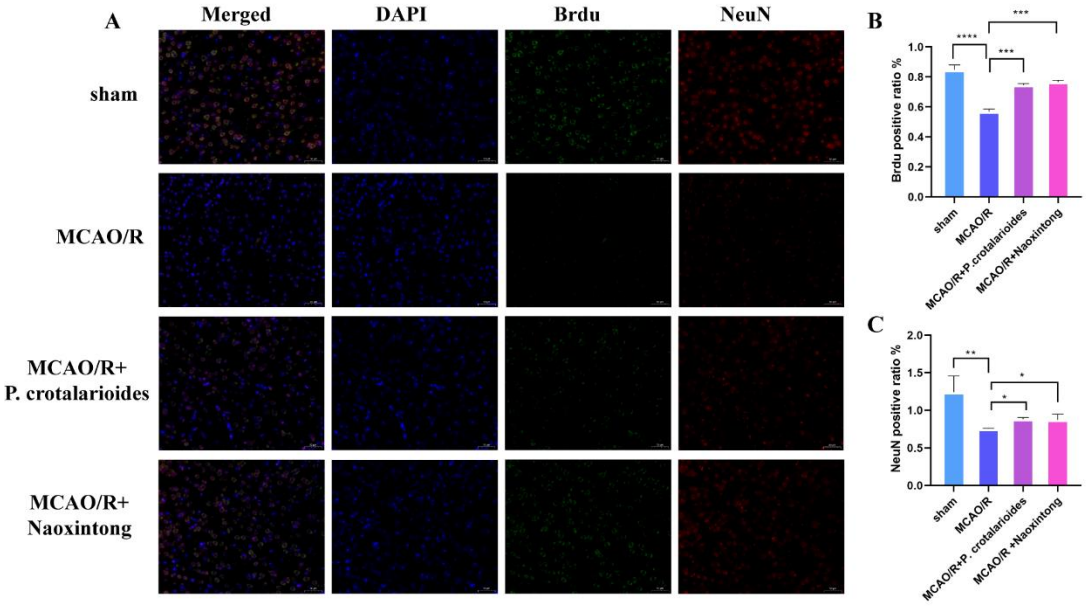

Fig.3. P. crotalarioides promoted the proliferation of neurons in vivo. A: Representative photomicrographs of the immunofluorescent staining of Brdu+ (red flurescence), NeuN+ (green fluorescence) and DAPI (blue fluorescence) in neurons. B: fluorescence intensity of Brdu. C: fluorescence intensity of NeuN. (Error bars represent mean ± SD, Magnification： 400×; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.)

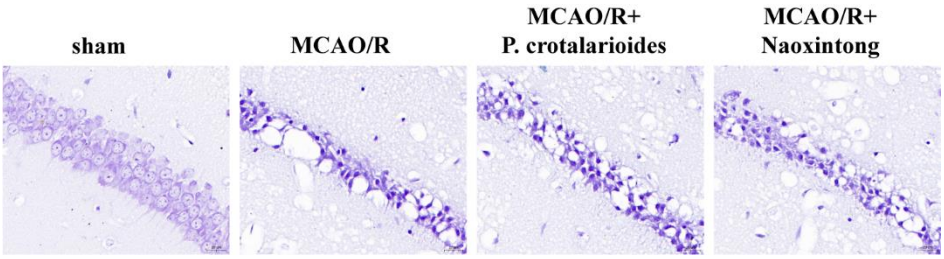

fig.4. P. crotalarioides alleviated the neuropathic damage under MCAO/R. Nissl staining in the sham group, MCAO/R group, MCAO/R + P. crotalarioides group, and the MCAO/R+Naoxintong group.