Effect of Chinese herbal formulae (BU-SHEN-YI-QI Granule) pretreatment on the dynamic expression of thrombin in NVU after ischemia/reperfusion

Aihua Liu
Huashan Hospital Affiliated to Fudan University
Jing Sun
Huashan Hospital Affiliated to Fudan University
Sagun Tiwari
Seventh People's Hospital of Shanghai University of TCM
Zhenxiang Han (hanxiang798007@gmail.com)
Seventh People's Hospital of Shanghai University of TCM

Research Article

Keywords: Bu-Shen-Yi-Qi granules (BSYQG), cerebral ischemia-reperfusion injury, neurovascular unit, thrombin

Posted Date: April 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1553761/v1

Abstract

Background

In a previous study, we demonstrated that Bu-Shen-Yi-Qi granule, a traditional Chinese herbal medicine compound, exhibits anti-inflammatory and neuroprotective properties in a rat model of ischemic stroke. In the present study, we examine whether Bu Shen Yi Qi granule (BSYQG) treatment has a protective impact on the dynamic expression of thrombin in the neurovascular unit of the ischemic brain following cerebral ischemia/reperfusion.

Methods

Adult male SD rats (n = 36) were divided into four groups: sham operation, ischemia/reperfusion (I/R), I/R + BSYQG-treatment (6g/kg/day), I/R + Argatroban (0.56 mg/ml). We established the ischemic/reperfusion model by classic cerebral artery occlusion (MCAO) for 1.5h, followed by 24h reperfusion. Immunofluorescence and TUNEL Double Staining were used to determine cell apoptosis in NVU. Immunofluorescence was used to examine the dynamic expression of thrombin in NVU.

Results

In cerebral ischemic/reperfusion damage, BSYQ treatment protected cells against apoptosis in the neurovascular unit. Furthermore, 6 hours after ischemic/reperfusion damage, BSYQ treatment significantly reduced brain thrombin activity.

Conclusion

BSYQ exerts protective effects in NVU after cerebral ischemic/reperfusion injury by inhibiting dynamic expression of thrombin.

1. Background

Acute ischemic stroke is one of the leading causes of disability and mortality around the world. According to the global burden of diseases from 1990–2016, stroke ranks first among all neurological disorders [1]. Clinically, with the success of thrombolytic clinical trials, medical science has made a breakthrough in stroke management [2]. Despite effective recanalization in stroke therapy, many stroke patients remain profoundly disabled. Functional independence rates are slightly under 50% even after extremely successful reperfusion therapies [3, 4], highlighting an unmet therapeutic need for supplementary neuroprotective treatments for reperfusion injury [5]. In an ischemia/reperfusion injury (I/RI) model, despite successful reperfusion, infarcts can grow. However, the mechanism of reperfusion injury is less evident in the human brain than in the experimental models [6]. Platelets may play a key role in cerebral

ischemia/reperfusion injury. Platelet degranulation and activated platelet receptors also may lead to subsequent infarct growth [7, 8]. Enhanced platelet activation may play an essential role in the development of ischemic stroke. Therefore, understanding the mechanism of platelet's contribution to cerebral ischemic injury may contribute to the development of a potential strategy in translation medicine.

In acute and chronic stages of ischemic stroke, platelet activation may increase the risk of subsequent cerebrovascular events [9]. Emerging evidence suggests that thrombin is a key hemostatic process regulator that activates platelets and plays a vital role in ischemic stroke via the activation of cellular protease-activated receptor (PAR) [10]. In ischemic stroke, thrombin is increased following blood-brain barrier disruption [11]. Subsequently, intravascular activation of thrombin causes brain edema and increases the ischemia region by acting on PARs, which are expressed in neuron and glial cells [5, 12, 13].

The growing recognition of thrombin's role in ischemic stroke offers a viable therapeutic implication. Argatroban is a recently approved direct thrombin inhibitor for the treatment of ischemic stroke [14, 15]. According to emerging evidence, argatroban treatment for ischemic stroke is safe and effective [16–18].In addition, argatroban did not enhance the risk of bleeding in the acute phase of cerebral infarction, according to the results of a meta-analysis [14]. However, argatroban's efficacy in non-cardioembolic stroke was not greater than that in cardioembolic stroke [19, 20].

Our previous studies have shown that Bu-Shen-Yi-Qi (BSYQ), a three-herb complex traditional Chinese herbal medicine, has anti-inflammatory and neuroprotective effects [21–23]. BSYQ significantly improved neurobehavioral dysfunction, alleviated neuronal damage, and inhibited neuronal pyroptosis [21–23]. High-dose treatment with 6g/kg Bu-Shen-Yi-Qi (BSYQ), but not 0.6 or 3g/kg BSYQ, showed the most significant effects in our previous study [24]. Furthermore, our earlier studies have shown that raw Radix rehmanniae, the primary component of BSYQ, can protect nerve cells in vitro by inhibiting thrombin [25–27]. In this study, we evaluated the effect of BSYQ on thrombin dynamic expression on the neurovascular unit (NVU) in a rat model of cerebral ischemia/reperfusion.

2. Materials And Methods

2.1 Animals

A total of 36 adult male Sprague-Dawley (SD) rats (240 ~ 270g) purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) were housed under specific pathogen-free conditions and given ad libitum access to water and food for 3 days. The Institutional Animal Care and Use Committee (IACUC) at Fudan University in China authorized the animal study.

2.2 Drugs and reagents

BSYQG is comprised of three traditional Chinese herbal medicines: Astragalus membranaceus (Huangqi), Epimedium brevicornu Maxim (Yinyanghuo), and Rehmannia glutinosa (Dihuang), all of which were

obtained as herbal granules from Jiangyin Tianjiang Pharmaceutical Co. Ltd. (Jiangyin, China). Dried Astragalus membranaceus, Epimedium brevicornu Maxim, and rehmannia glutinosa were immersed in 10 times of water for 2h, boiled for 1h, filtered, and sprayed dried into granules. These herbal granules were stored at 4°C with a ratio of 3:2:1.5 at normal saline before use. The final concentration of oral administration for the animal is set as 6g/kg daily(10 times doses for adults).

As a positive control, argatroban, a direct thrombin inhibitor that lowers brain damage following ischemic stroke, was used [13]. Rats were administrated by IV argatroban injection (10mg/kg dose dissolved in 0.9% saline at 0.56mg/ml concentration) at 0.5h, 1h, 2h, 4h, and 6h after ischemic/reperfusion [28].

Immunofluorescence was performed using mouse monoclonal antibodies for NeuN and thrombin from Abcam (Shanghai, China), GFAP from Proteintech (Wuhan, China), CD31 from Abcam (Cambridge, USA), Alexa Fluor 555 labeled donkey anti-rabbit IgG(H + L), and Alexa Fluor 555 labeled donkey anti-mouse IgG(H + L) from Beyotime (Shanghai, China).

2.3 Model of middle cerebral artery occlusion/reperfusion (MCAO/R)

The MCAO/R rat model was developed using a method of reversible regional I/R injury [27]. Rats were anaesthetized intraperitoneally with 10% chloral hydrate (0.35mL/100g body weight) prior to operation. During the neck incision, the left common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were isolated and exposed. The embolus created from the nylon filament was then used to occlude the MCA through a tiny incision in the internal carotid artery. After 90 minutes, a filament was removed to allow for 24 hours of reperfusion (Fig. 1A). The rats in the I/R, BSYQG, and argatroban groups were given brain ischemia and reperfusion, whereas the sham-operated animals were given an identical treatment but without artery ligation.

The effectiveness of the MCAO/R model in rats was validated by neurobehavioral scores. During the surgery, the rat's body temperature and respiration rate were monitored.

2.4 Experimental Design

Adult male SD rats were randomly assigned to one of four groups, each with six members: sham operation, ischemia/reperfusion (I/R), I/R + BSYQG-treatment (6 g/kg/day), and I/R + Argatroban (0.56 mg/ml). Oral gavage was used to provide medicines to the rats. The I/R + BSYQG rats were given BSYQG at a rate of 6 g/kg/day orally, while the sham and I/R rats were given the same double-distilled water (0.1ml/kg/day) for 7 days by oral gavage. I/R + Argatroban rats were administered Argatroban at doses of 0.56 mg/kg 12 hours and 1 hour before ischemia induction.

2.5. Immunofluorescence and TUNEL Double Staining

TdT-mediated dUTP Nick-End labelling (TUNEL) and immunofluorescence labelling were used to examine cerebral ischemia and reperfusion-induced cell apoptosis in NVU, as previously described. Anti-NeuN, anti-GFAP, and anti-CD31 antibodies were utilized to stain neurons, astrocytes, and vascular endothelial cells

with a TUNEL kit (Roche Diagnostics GmbH, 11684817910, Penzberg, Germany). The brain slices were then blocked with goat serum for 1 hour before being treated with the primary antibody overnight at 4°C. The slices were rinsed in PBS for 5 minutes 3 before being incubated with secondary antibodies for 60 minutes at 37°C in a light-shielded environment. For 15 minutes, the slices were stained with DAPI (Beyotime, C1002, China). For TUNEL staining, the slices were incubated in a dark humidified chamber at 37°C for 1 hour with TUNEL reaction mixture, followed by a final wash for 10 minutes 3 with PBS and then covered with glycerine. A fluorescent microscope was used to examine the recorded pictures (NIKON, ECLIPSE Ni, Japan). An investigator who was unaware of the experimental groups computed positive cells at random throughout the five lesion locations in the ischemic brain. Apoptotic cells (TUNEL-positive cells) were compared to NeuN for neurons, GFAP for astrocytes, and CD31 for vascular endothelial cells to determine an apoptosis ratio.

2.6. Expression of thrombin after MCAO

The expression of thrombin in each group was observed dynamically by immunofluorescence (0.5h, 1h, 2h, 4h, 6h after MACAO). Two paraffin sections were taken from the infarcted basal ganglia brain tissue at each time point in each rat, placed in a constant temperature oven at 65°C for 30 min, and then immersed in xylene I and II for 15 min. Waxed parts were soaked for 5 minutes in various concentrations of alcohol (100%, 95%, 85%, 75%) before being rinsed for 10 minutes with tap water. After natural cooling, repair with high pressure in 0.01M sodium citrate buffer solution for 15 minutes, then wash three times with 0.02M PBS, microwave heating in "3-5-3 method", and heat 0.01M sodium citrate buffer in an electric furnace or water bath (pH6.0) to about 95°C, after which place the slices in the slices and heat them for 10–15 minutes for boiling repair. The primary antibody (Thrombin) was added dropwise and incubated at 4°C overnight; the fluorescent secondary antibody was added dropwise, placed at room temperature for 1 h, the anti-quenching mounting medium and DAPI were diluted 1:500 and mounted, stored at -20°C, and filmed under a fluorescence microscope until a total of Observe and collect images under a focusing microscope. The integrated optical density (IOD) of thrombin was calculated using ImagePro Plus 6 software.

2.7. Statistical Analysis.

GraphPad Prism 8.0.1 was used to conduct the statistical analysis (SanDiego,CA). All data were presented as mean ± SD. Statistically significant correlation was assessed by one-way ANOVA followed by an LSD comparison test or unpaired Student's t-tests using SPSS 11.5 for Windows (Chicago, IL, USA). A statistically significant difference was considered as $P<0.05$.

3. Results

3.1. BSYQG treatment protected from apoptosis in the neurovascular unit in rats after MCAO

The co-expression of TUNEL and immunofluorescence were calculated to identify apoptotic neuron cells (NeuN), astrocytes (GFAP), and vascular endothelial cells(CD31). A few apoptotic cells were observed in the brain cortex in the Sham group. Furthermore, the number and rate of apoptotic neuron cells, astrocytes, and endothelial cells significantly increased in the brain cortex after cerebral ischemic-reperfusion in the ischemic cortex compared with the sham group (neuron cells apoptosis $P = 0.000 < 0.001$, astrocyte apoptosis $P = 0.004 < 0.001$, vascular endothelial cells $P = 0.001 < 0.001$.).BSYQG and argatroban treatment significantly attenuated apoptosis and showed a reduction in the numbers of cell apoptosis (BSYQG group vs I/R group: neuron cells apoptosis $P = 0.001 < 0.01$, astrocyte apoptosis $P = 0.017 < 0.05$, vascular endothelial cells $P = 0.000 < 0.01$, argatroban group vs. I/R group: neuron cells apoptosis $P = 0.000 < 0.01$, vascular endothelial cells $P = 0.001 < 0.05$).It is a significant reduction in the rate of cells apoptosis in the treatment groups(BSYQG group vs. I/R group: neuron cells apoptosis $P = 0.001 < 0.01$, vascular endothelial cells $P = 0.009 < 0.01$. argatroban group vs I/R group: neuron cells apoptosis $P = 0.000 < 0.01$).

3.2. The expression of thrombin in both cerebral cortex after cerebral ischemia-reperfusion injury

After ischemia in the middle cerebral artery of rats, with the prolongation of ischemia time (0.5,1,2,4,6 h), the content of thrombin in the brain showed a curve, where it initially decreased (0.5h), then increased (4h), and finally decreased (6h) in the cerebral cortex. Thrombin content in the left brain was significantly higher at 4 h after ischemia-reperfusion than 1h ($P = 0.003$) and 2h ($P = 0.003$)after ischemia-reperfusion. However, there was no significant difference in the right brain during ischemia-reperfusion.

3.3. BSYQG treatment decreased the expression and activity of thrombin in rats after MCAO

After an ischemic stroke, increased levels of thrombin expression and activity are linked to brain lesions. The results indicated that after 2 hours ($P = 0.032$), 4 hours ($P = 0.000$), and 6 hours of MCAO ($P = 0.008$), the levels of thrombin in the I/R group were considerably higher than those in the sham group in the left ischemic brain. At 4 hours after MCAO, treatment with BSYQG ($P = 0.001$) and argatroban ($P = 0.003$) significantly decreased brain thrombin activity. Furthermore, treatment with BSYQG ($P = 0.043$) significantly decreased brain thrombin activity 6 hours after MCAO. Then, in each time period, there was no significant difference among the groups in the right brain.

4. Discussion

The current study has found that BSYQG treatment significantly reduces apoptosis of neurons, astrocytes, and endothelial cells in the ischemic brain after MCAO. So, it can be protected from apoptosis in the neurovascular unit in rats after MCAO. We also showed that the expression of thrombin was upregulated after 2h of ischemic/reperfusion. Importantly, BSYQG treatment significantly decreased brain thrombin activity at 4h and 6h after MCAO. Our study demonstrates that the treatment of BSYQG on the MCAO animal model plays a pivotal role in protecting the NVU by inhibiting the thrombin.

The NVU is a multicellular and multi-component network that is necessary for brain health and equilibrium, and its disturbance is the prime pathophysiological mechanism of ischemic stroke. Thrombin plays a vital role in the mechanisms and pathways with brain injury following ischemic stroke [11]. In ischemic stroke, the higher expression of thrombin is due to breakdown blood-brain-barrier (BBB) [29] and facilitates severe neurovascular unit injury [13]. High thrombin concentration also has a role in brain injury, particularly in the production of cerebral edema and the expansion of the ischemic region after a stroke [30]. It can cleave fibrinogen into insoluble fibrin to generate thrombus, is involved in the death and survival of brain cells, and may act as a "gas pedal" in hemostasis [31–33]. Furthermore, neuronal edema, excitotoxicity, and neuroinflammatory can cause neuron and astrocyte death followed by the high concentration of thrombin after ischemic stroke [34]. This study found that the number of apoptotic neurons, astrocytes, and endothelial cells in the brain cortex rose considerably following cerebral ischemia-reperfusion in the ischemic cortex compared to the sham group. Also, the quantity of thrombin in the ischemic brain was significantly higher at 4 hours than at 0.5 and 6 hours following ischemia/reperfusion.

Bu-Shen-Yi-Qi (BSYQ), comprised of three traditional Chinese herbal medicines: Astragalus membranaceus (Huangqi), Epimedium brevicornu Maxim (Yinyanghuo), and Rehmannia glutinosa (Dihuang), is a compound traditional Chinese herbal prescription used to be proved as anti-inflammatory and neuroprotective effects [21–23]. In the previous study of the effect of BSYQ components, the following chemical components were extracted from BSYQ: Catalpol, Leonuride, Calycosin-7-o-β-D-glucoside, Hyperoside, Acteoside, Formononetin-7-o-β-D-glucoside, Epimedin A, Calycosin, Epimedin B, Epimedin C, Icariin, Formononetin, Astragaloside IV, Astragaloside II, Baohuoside-I, Astragaloside I [35]. The primary chemical constituents in the effective BSYQ were determined by HPLC. The highest concentrations were Icariin and epimedin C followed by catalpol, epimedin B, astragaloside IV, and baohuoside-I [35]. Icarlin is a key active ingredient of Epimedium grandiflorum, a traditional Chinese medicinal plant. Icarlin has been shown to have a neuroprotective effect, preventing brain damage caused by ischemia/reperfusion [36, 37]. Catapol, the main component of Rehmannia glutinosa, can ameliorate impaired neurovascular units in the ischemic region and promote angiogenesis to replenish lost vessels and neurons [38]. Astragaloside IV, a primary bioactive compound of Radix Astragali, could decrease the neurological score, reduce the brain's infarct volume, and alleviate cerebral injury after cerebral injury [39]. According to our previous study, the two dosages of BSYQ (3g/kg, 6g/kg) enhanced neurological dysfunction, lowered the extent of cerebral infarction, boosted neuron survival, and decreased neuron apoptosis following cerebral I/R [24]. There was also a dose-response association between the low and high doses. In the study, we found BSYQ (6g/kg) treatment could significantly attenuate cell apoptosis in NVU and protect neurons and astrocytes in the ischemic brain. Furthermore, 6 hours after MCAO, BSYQG treatment significantly lowered brain thrombin activity.

In conclusion, we have demonstrated that BSYQ can inhibit thrombin activity and hence ameliorate ischemic I/R injury in the NVU. In addition, a traditional Chinese medicine compound prescription is a mixture of multiple traditional medicines, and its chemical composition can impact the total medicinal

effect by not only enhancing but also limiting the effects of each other. As a result, the protective mechanisms of BSYQ on ischemic injury remain to be further clarified.

Declarations

Ethics approval :

The Institutional Animal Care and Use Committee (IACUC) at Fudan University in China authorized the animal study.

Availability of data and material:

Data/Reports are *available* from the authors upon reasonable *request* .

Funding:

This work was supported by the National Natural Science Foundation of China(No.81703997), the Outstanding Leaders Training Program of Pudong Health Bureau of Shanghai , Natural Science Foundation of Shanghai(Grant No.21ZR1449300),Clinical Research Program of Shanghai science and technology committee(Grant No.21Y11921000).

Competing interests:

The authors declare that they have no competing interests.

Author's Contribution:

Aihua Liu and Zhenxiang Han are the principal investigator of the study, overseeing study design, data collection and manuscript approval. Jing Sun initiated the study. Sagun Tiwari is the coinvestigators who drafted and critically revised the manuscript for important intellectual content. All authors contributed to the refinement of the study and approved the final manuscript.

Acknowledgments: Nil

References

1. **Global, regional, and national burden of neurological disorders, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016**. Lancet Neurol 2019, **18**(5):459–480.
2. **Tissue plasminogen activator for acute ischemic stroke**. N Engl J Med 1995, **333**(24):1581–1587.
3. Albers GW, Marks MP, Kemp S, Christensen S, Tsai JP, Ortega-Gutierrez S, McTaggart RA, Torbey MT, Kim-Tenser M, Leslie-Mazwi T *et al*: **Thrombectomy for Stroke at 6 to 16 Hours with Selection by Perfusion Imaging**. N Engl J Med 2018, **378**(8):708–718.

4. Goyal M, Menon BK, van Zwam WH, Dippel DW, Mitchell PJ, Demchuk AM, Dávalos A, Majoie CB, van der Lugt A, de Miquel MA *et al*: **Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomised trials**. Lancet 2016, **387**(10029):1723–1731.
5. Stegner D, Klaus V, Nieswandt B: **Platelets as Modulators of Cerebral Ischemia/Reperfusion Injury**. Front Immunol 2019, **10**:2505.
6. Gauberti M, Lapergue B, Martinez de Lizarrondo S, Vivien D, Richard S, Bracard S, Piotin M, Gory B: **Ischemia-Reperfusion Injury After Endovascular Thrombectomy for Ischemic Stroke**. Stroke 2018, **49**(12):3071–3074.
7. Prévost N, Woulfe DS, Jiang H, Stalker TJ, Marchese P, Ruggeri ZM, Brass LF: **Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets**. Proc Natl Acad Sci U S A 2005, **102**(28):9820–9825.
8. Stegner D, Nieswandt B: **Platelet receptor signaling in thrombus formation**. J Mol Med (Berl) 2011, **89**(2):109–121.
9. Lieschke F, Zheng Y, Schaefer JH, van Leyen K, Foerch C: **Measurement of Platelet Function in an Experimental Stroke Model With Aspirin and Clopidogrel Treatment**. Front Neurol 2020, **11**:85.
10. Brailoiu E, Shipsky MM, Yan G, Abood ME, Brailoiu GC: **Mechanisms of modulation of brain microvascular endothelial cells function by thrombin**. Brain Res 2017, **1657**:167–175.
11. Ye F, Garton HJL, Hua Y, Keep RF, Xi G: **The Role of Thrombin in Brain Injury After Hemorrhagic and Ischemic Stroke**. Transl Stroke Res 2021, **12**(3):496–511.
12. van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, van Hinsbergh VW: **Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases**. Circ Res 2000, **87**(4):335–340.
13. Chen B, Cheng Q, Yang K, Lyden PD: **Thrombin mediates severe neurovascular injury during ischemia**. Stroke 2010, **41**(10):2348–2352.
14. Lv B, Guo FF, Lin JC, Jing F: **Efficacy and safety of argatroban in treatment of acute ischemic stroke: A meta-analysis**. World J Clin Cases 2022, **10**(2):585–593.
15. Hou X, Jin C, Pan C, Wang X, Xue J, Yang Z, Qi D: **Effects of argatroban therapy for stroke patients: A meta-analysis**. J Clin Neurosci 2021, **90**:225–232.
16. Barreto AD, Ford GA, Shen L, Pedroza C, Tyson J, Cai C, Rahbar MH, Grotta JC: **Randomized, Multicenter Trial of ARTSS-2 (Argatroban With Recombinant Tissue Plasminogen Activator for Acute Stroke)**. Stroke 2017, **48**(6):1608–1616.
17. Berekashvili K, Soomro J, Shen L, Misra V, Chen PR, Blackburn S, Dannenbaum M, Grotta JC, Barreto AD: **Safety and Feasibility of Argatroban, Recombinant Tissue Plasminogen Activator, and Intra-Arterial Therapy in Stroke (ARTSS-IA Study)**. J Stroke Cerebrovasc Dis 2018, **27**(12):3647–3651.
18. Yang Y, Zhou Z, Pan Y, Chen H, Wang Y: **Randomized trial of argatroban plus recombinant tissue-type plasminogen activator for acute ischemic stroke (ARAIS): Rationale and design**. Am Heart J 2020, **225**:38–43.

19. Wada T, Yasunaga H, Horiguchi H, Matsubara T, Fushimi K, Nakajima S, Yahagi N: **Outcomes of Argatroban Treatment in Patients With Atherothrombotic Stroke: Observational Nationwide Study in Japan**. Stroke 2016, **47**(2):471–476.
20. Oguro H, Mitaki S, Takayoshi H, Abe S, Onoda K, Yamaguchi S: **Retrospective Analysis of Argatroban in 353 Patients with Acute Noncardioembolic Stroke**. J Stroke Cerebrovasc Dis 2018, **27**(8):2175–2181.
21. Luo QL, Nurahmat M, Li MH, Sun J, Chen MX, Liu F, Wei Y, Dong JC: **Pharmacological investigation of a HPLC/MS standardized three herbal extracts containing formulae (Bu-Shen-Yi-Qi-Tang) on airway inflammation and hypothalamic-pituitary-adrenal axis activity in asthmatic mice**. Phytomedicine 2014, **21**(11):1439–1450.
22. Wei Y, Luo QL, Sun J, Chen MX, Liu F, Dong JC: **Bu-Shen-Yi-Qi formulae suppress chronic airway inflammation and regulate Th17/Treg imbalance in the murine ovalbumin asthma model**. J Ethnopharmacol 2015, **164**:368–377.
23. Qin J, Wuniqiemu T, Wei Y, Teng F, Cui J, Sun J, Yi L, Tang W, Zhu X, Xu W *et al*: **Proteomics analysis reveals suppression of IL-17 signaling pathways contributed to the therapeutic effects of Jia-Wei Bu-Shen-Yi-Qi formula in a murine asthma model**. Phytomedicine 2022, **95**:153803.
24. Liu A SJ, Kong L, Han Z, Dong Z: **Effect of Replenishing Kidney and Strengthening Qi formula on PI3K/AKT Pathway in MCAO Rats**. *Zhonghua Zhongyiyao Zazhi* 2022, **In press**.
25. Zhou X WZ, Li W, Wei J: **Experimental study on apoptosis of astrocytes induced by antithrombin of Shengji Injection**. Chinese Journal of Integrative Medicine on Cardio-Cerebrovascular Disease 2012;10: 1098–9.
26. Liu A LH: **Experimental study of the protective effects of Shengdi injection on the neuron of stroke model**. Journal of Tongji University(Medical Science) 2009;30: 52–4.
27. Liu A YX, Wang Y, Wei J: **An experimental study of protective effects of Shengdi injection on neuron injured by thrombin**. Chinese journal of integrated traditional and western medicine in intensive and critical care 2008;03: 155–8.
28. Singh S, Houng AK, Wang D, Reed GL: **Physiologic variations in blood plasminogen levels affect outcomes after acute cerebral thromboembolism in mice: a pathophysiologic role for microvascular thrombosis**. J Thromb Haemost 2016, **14**(9):1822–1832.
29. Krenzlin H, Lorenz V, Danckwardt S, Kempski O, Alessandri B: **The Importance of Thrombin in Cerebral Injury and Disease**. Int J Mol Sci 2016, **17**(1).
30. Sokolova E, Reiser G: **Prothrombin/thrombin and the thrombin receptors PAR-1 and PAR-4 in the brain: localization, expression and participation in neurodegenerative diseases**. Thromb Haemost 2008, **100**(4):576–581.
31. Petzold T, Massberg S: **Thrombin: A Gas Pedal Driving Innate Immunity**. Immunity 2019, **50**(4):1024–1026.
32. Delvaeye M, Conway EM: **Coagulation and innate immune responses: can we view them separately?** *Blood* 2009, **114**(12):2367–2374.

33. Fenton JW, 2nd: **Thrombin**. Ann N Y Acad Sci 1986, **485**:5–15.
34. Rajput PS, Lamb J, Kothari S, Pereira B, Soetkamp D, Wang Y, Tang J, Van Eyk JE, Mullins ES, Lyden PD: **Neuron-generated thrombin induces a protective astrocyte response via protease activated receptors**. Glia 2020, **68**(2):246–262.
35. Nurahmat M, Chen M, Luo Q, Ling Y, Dong J, Huang C: **Rapid characterization and determination of multiple components in Bu-Shen-Yi-Qi-Fang by high-performance liquid chromatography coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry**. J Sep Sci 2014, **37**(23):3509–3517.
36. He C, Wang Z, Shi J: **Pharmacological effects of icariin**. Adv Pharmacol 2020, **87**:179–203.
37. Xiong D, Deng Y, Huang B, Yin C, Liu B, Shi J, Gong Q: **Icariin attenuates cerebral ischemia-reperfusion injury through inhibition of inflammatory response mediated by NF-κB, PPARα and PPARγ in rats**. Int Immunopharmacol 2016, **30**:157–162.
38. Wang HJ, Ran HF, Yin Y, Xu XG, Jiang BX, Yu SQ, Chen YJ, Ren HJ, Feng S, Zhang JF *et al*: **Catalpol improves impaired neurovascular unit in ischemic stroke rats via enhancing VEGF-PI3K/AKT and VEGF-MEK1/2/ERK1/2 signaling**. Acta Pharmacol Sin 2021.
39. Zhang Y, Zhang Y, Jin XF, Zhou XH, Dong XH, Yu WT, Gao WJ: **The Role of Astragaloside IV against Cerebral Ischemia/Reperfusion Injury: Suppression of Apoptosis via Promotion of P62-LC3-Autophagy**. Molecules 2019, **24**(9).

Figures

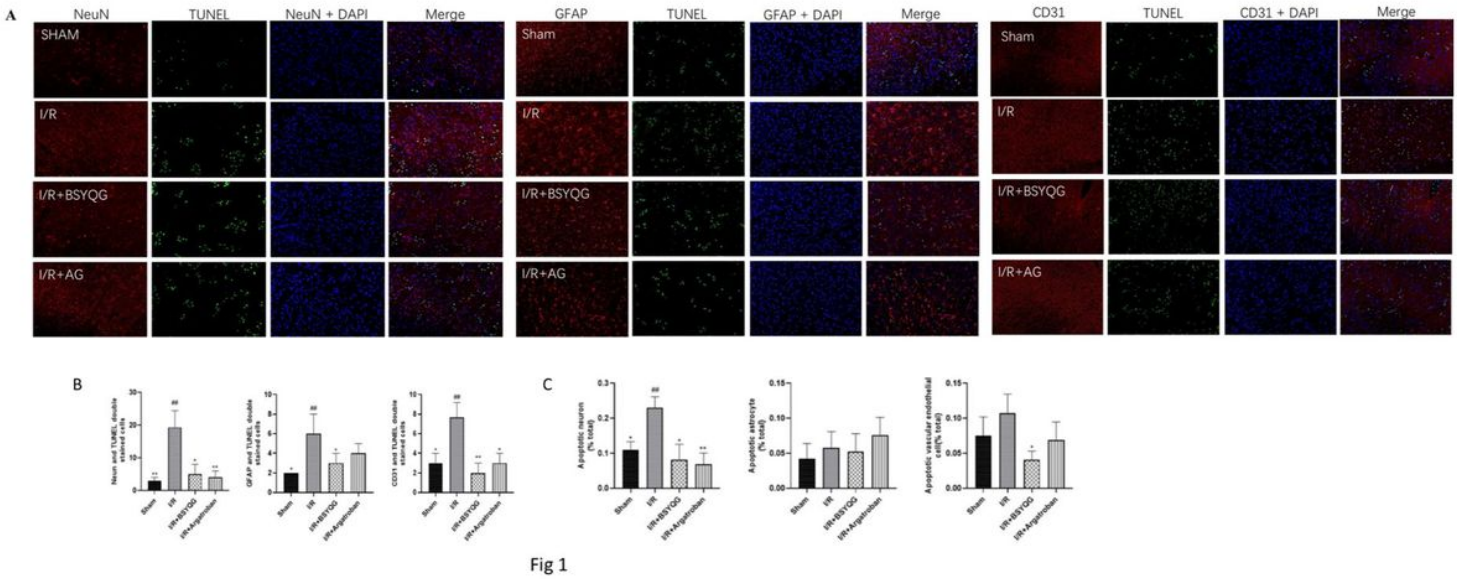

Figure 1

Representative images of apoptosis of different groups in the NVU of ischemic cortex 24h after reperfusion. (A) Representative photomicrographs of immunofluorescence labeling with NeuN

(red)/GFAP(red)/CD31(red) and TUNEL (green) double staining. Nuclei were counterstained with DAPI (blue), and collocation of green and blue indicated TUNEL positive cells. Scale Bar=50μm(B) Quantification of apoptotic cells. TUNEL and NeuN/GFAP/CD31 double-stained cells (yellow) indicated the apoptotic neurons. (C) Percentage of apoptotic cells in NeuN/GFAP/CD31 positive cell. Data are reported as the means ± SD. n=3; #P<0.05 vs the sham group,## P < 0.001 vs the sham group,$*P$ < 0.05 vs the I/R group, $**P$ < 0.001 vs the I/R group.

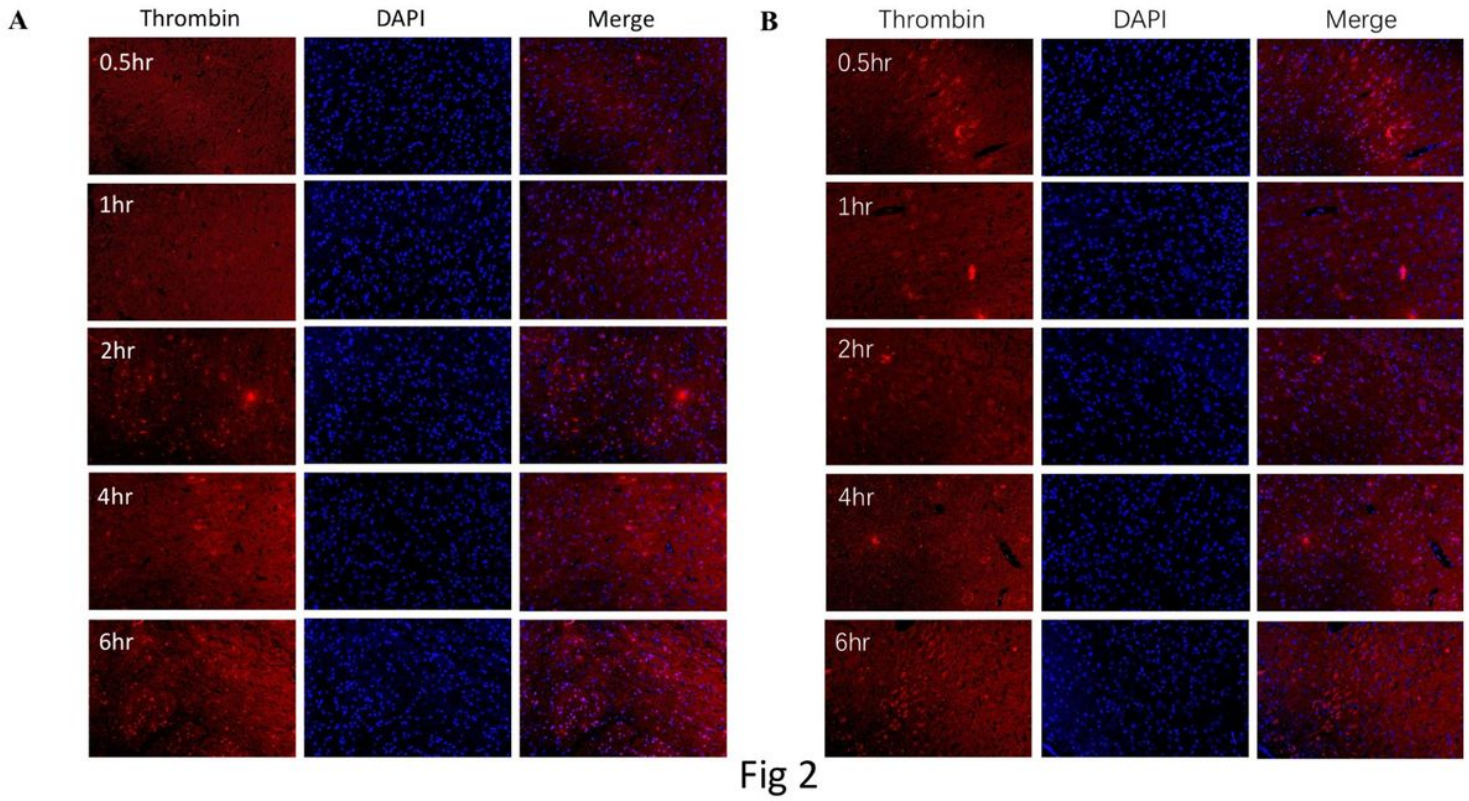

Fig 2

Figure 2

the expression of thrombin changed in both cortex of MCAO rats. (A-B) The immunofluorescence labeling with thrombin (red) and Nuclei were counterstained with DAPI (blue) in the ipsilateral ischemic cerebral cortex(A) and the contralateral cerebral cortex(B). Scale Bar=50μm. Dynamic changes of thrombin antigen were found in the ipsilateral ischemic cerebral cortex(C) and the contralateral cerebral cortex(D). The values were presented as mean ± standard error of the mean (n=3) in the left cerebral hemisphere. **P < 0.01

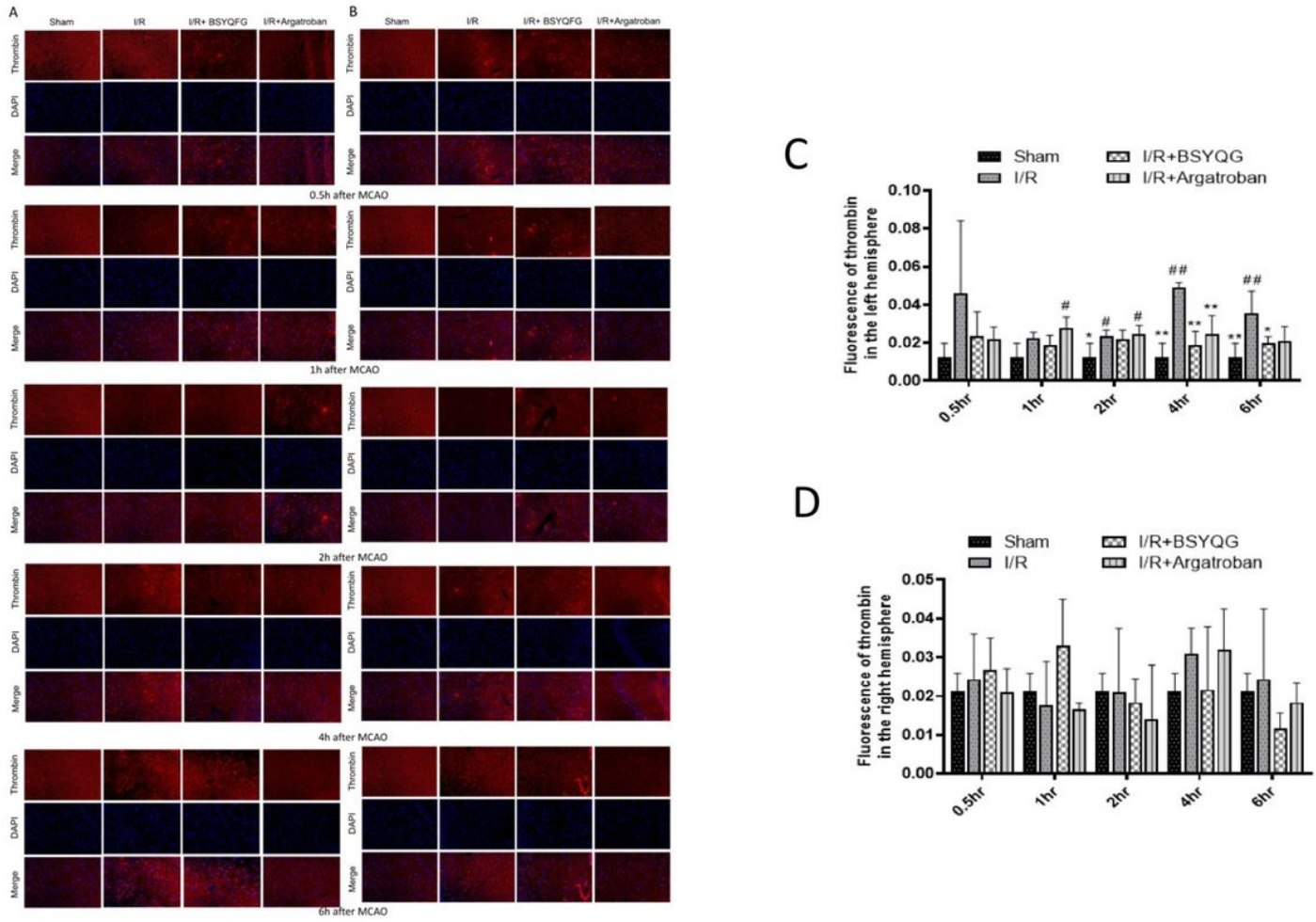

Figure 3

(A-B) Representative images of fluorescence expression of thrombin in the left(ischemic brain,A) and right(normal brain,B) cerebral hemisphere in different time points. Representative photomicrographs of immunofluorescence labeling with thrombin (red) and Nuclei were counterstained with DAPI (blue) after MCAO 0.5h,1h,2h,4h 6h. (C-D)The dynamic percentage of thrombin were expressed in regions of the left(C) and right(D) cerebral hemisphere.

The values were presented as mean ± standard error of the mean (n =3). #$P < 0.05$, vs Sham group; *$P < 0.05$,* *$P < 0.01$ vs I/R group.