Combination of puerarin and tanshinone IIA alleviates ischaemic stroke injury in rats via activating the Nrf2/ARE signalling pathway

Qing Miao, Ruihai Wang, Xiaoxin Sun, Song Du and Limei Liu

China Academy of Chinese Medical Sciences, Institute of Basic Theory for Chinese Medicine, Beijing, China

ABSTRACT

Context: Puerarin (Pue) and tanshinone IIA (Tan IIA) are often used in combination in the treatment of cerebrovascular diseases.

Objective: To investigate the neuroprotective effect and synergic mechanism of Pue-Tan IIA on the treatment of ischaemic stroke (IS).

Materials and methods: IS was induced in rats by middle cerebral artery occlusion (MCAO). Rats were intraperitoneally injected with Pue (36 mg/kg), Tan IIA (7.2 mg/kg), or Pue-Tan IIA (36 and 7.2 mg/kg) for five times [30 min before ischaemia, immediately after reperfusion (0 h), 24, 48, and 72 h after reperfusion]. After administration, neurological function assessment and histological changes in the brain were performed. S-100β and NSE levels were measured to determine the severity of brain injury. Oxidative stress parameters and inflammatory mediators were measured. The proteins involved in Nrf2/ARE signalling pathway were determined by qRT-PCR and western blot.

Results: After administration, the neurological function scores, infarct volume, S-100β, and NSE levels were significantly reduced in MCAO rats, especially with Pue-Tan IIA treatment (p < 0.05). All treatments increased T-AOC, CAT, SOD, and GSH activities and reduced GSSG activity and MDA, IL-6, TNF-α, ICAM-1, and COX-2 levels in MCAO rats. Pue-Tan IIA significantly increased Nrf2 expression in the nucleus (1.81-fold) and decreased its expression in the cytoplasm (0.60-fold). Pue-Tan IIA significantly increased the expressions of HO-1 (1.87-fold) and NQO1 (1.76-fold) and decreased Keap1 expression (0.39-fold).

Discussion and conclusions: The combination of Pue and Tan IIA could alleviate ischaemic brain injury by activating Nrf2/ARE signalling pathway, providing an experimental basis for clinical applications.

Introduction

Ischaemic stroke (IS) is a common acute cerebrovascular disease and one of the principal causes of death worldwide. It is characterized by high morbidity, disability, lethality, and recurrence. With the ensuing irreversible brain damage and loss of neuronal function, IS poses a great threat to human health and life, as well as a considerable economic and social burden (Gorelick et al. 2019). Current approaches to treating stroke as well as a considerable economic and social burden (Gorelick et al. 2019). Current approaches to treating stroke include administration of tPA and salvage therapy. However, these treatments are limited by their narrow therapeutic windows and high toxicity. Therefore, the development of novel therapeutic strategies is urgently needed.

Neuroprotective therapy has become the key to effective prevention and treatment of stroke in recent years. Among the medications evaluated for stroke therapy, phytochemicals, the most important resources for the development of new drugs, have shown great neuroprotective potential in preclinical research (Xu et al. 2021). Additionally, combination therapy using phytochemicals can address multiple targets and various pathways through synergistic mechanisms (Fisher 2011). Therefore, exploiting the novel combination therapy of phytochemicals is highly imperative for IS.

Puerarin (Pue) and tanshinone IIA (Tan IIA) (Figure 1) are the main active components of Pueraria lobata (Willd.) Ohwi (Leguminosae) and Salvia miltiorrhiza Bge. (Lamiaceae), respectively. They are the most used herbal drugs in China. The combination of the two is recorded in ‘Shijinmo Duiyao’ as a common herb pair for promoting blood circulation and removing blood stasis in the clinical treatment of IS (Lv 2016; Gao et al. 2019; Sun et al. 2020). Numerous studies have shown that Pue, a polyhydroxy isoflavonoid, can selectively accumulate in the ischaemic tissues of the brain, increase cerebral blood flow and vascular endothelial growth factor expression and inhibit the release and production of inflammatory factors, excitatory amino acid toxicity, and oxidative stress (Cheng et al. 2016; Liu et al. 2016; Xu et al. 2016; Kong et al. 2019). Tan IIA is a lipid-soluble derivative of phenanthrenequinone. Tan IIA has demonstrated antioxidant, anti-inflammatory, antibacterial, anti-apoptotic effects, and it can easily penetrate the blood–brain barrier (Chen et al. 2012; Li et al. 2015; Yang et al. 2016; Cai et al. 2017). Injection forms of Pue and Tan IIA have been developed and used clinically in the treatment of cerebrovascular diseases, respectively (Zheng et al. 2017; Zhang et al. 2020). Current clinical studies have shown that the combination of two treatments is more effective than a single injection for reducing the symptoms in patients with affective disorders after the first acute cerebral infarction, with a lower incidence of adverse reactions (Bai and Lv 2014). However, the exact neuroprotective effects and underlying mechanisms of Pue-Tan IIA on the treatment of IS remain unclear and need to be evaluated in depth.

Therefore, this study investigated the neuroprotective effect of the combination of Pue and Tan IIA on IS induced by middle
cerebral artery occlusion (MCAO) and further explored the underlying mechanism. Furthermore, the effects of Pue monotherapy, Tan IIA monotherapy, and Pue-Tan IIA therapy on IS were compared to verify the synergistic effect of the combination of Pue and Tan IIA on alleviating ischaemic brain injury.

Materials and methods
Animals and drugs
Male Sprague-Dawley rats (240 ± 20 g) were purchased from the Experimental Animal Centre of Hubei Province (Licence No. SCXK 2020-0018, Wuhan, China). Before experimentation, the rats were adapted to an environmentally controlled breeding room for 7 days. All experimental procedures were carried out in accordance with the guidelines of the Laboratory Animal Ethics Committee of the Institute of Basic Theory for Chinese Medicine, China Academy of Chinese Medical Sciences (No. 202020079, Beijing, China). Puerarin injection (2 mL:100 mg, 190402) was obtained from Guangzhou Baiyunshan Tianxin Pharmaceutical Co., Ltd. (Guangdong, China). Sulfotanshinone sodium injection (2 mL:10 mg, 1903115) was obtained from SPH No.1 Biochemical & Pharmaceutical Co., Ltd. (Shanghai, China). Nimodipine injection (50 mL:10 mg, BXJ3B71) was obtained from Bayer Healthcare Co., Ltd. (Beijing, China).

MCAO model
The experimental model was established by MCAO. The rats were anaesthetized and placed in a supine position. The centre area of the neck was incised, and the skin and muscle were separated bluntly. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were separated from the underlying connective tissue. ECA and CCA were ligated, and ICA was distally clamped with a microvascular clip. A nylon suture with a spherical tip was inserted through a small incision between ECA and ICA. The temporary clamp on the ICA was removed and the suture was slowly inserted up to 18 mm to occlude the blood flow to the middle cerebral artery. After ischaemia for 90 min, the suture was pulled out to allow reperfusion. The rats in the sham group were subjected to the same surgical procedure, minus the insertion of the suture.

Drug administration and groups
Rats were randomly divided into six groups: (1) sham group (control; n = 15); (2) MCAO group (model; n = 15); (3) nimodipine group (Nimo; MCAO rats injected with 1 mg/kg nimodipine, n = 15); (4) puerarin group (Pue; MCAO rats injected with 36 mg/kg puerarin, n = 15); (5) tanshinone IIA group (Tan IIA; MCAO rats injected with 36 mg/kg sulfotanshinone sodium, n = 15); and (6) puerarin-tanshinone IIA group (Pue-Tan IIA; MCAO rats injected with 36 mg/kg puerarin and 7.2 mg/kg sulfotanshinone sodium, n = 15). The treatments were administered in human equivalent doses. The sham and MCAO groups were given an equal volume of 5% glucose injection. Rats in each group were injected five times intraperitoneally: 30 min before ischaemia, immediately after reperfusion (0 h), 24, 48, and 72 h after reperfusion.

Neurological function assessment
Seventy-two hours after reperfusion, the neurological function assessments of all rats were performed using the Longa scoring method (Longa 1989). The rating scale was as follows: 0 point, no neurological deficits; 1 point, inability to fully extend the contralateral forepaw; 2 points, circling to the contralateral side; 3 points, falling to the contralateral side; 4 points, inability to walk spontaneously and with a depressed level of consciousness.

Triphenyl tetrazolium chloride staining
The brain tissues were frozen at −20°C for 20 min and then sliced into sections along the coronal plane. These slices were stained with triphenyl tetrazolium chloride (TTC) at 37°C for 15 min and transferred in 4% paraformaldehyde afterwards. After 24 h, the brain slices were removed and photographed. Image Pro Plus 6.0 software was used to measure the infarct volume. The percentage of infarction was calculated using the following formula: total infarct volume/total brain volume × 100%.

Hematoxylin-eosin and Nissl staining
After the neurobehavioral assessment, all rats were sacrificed after anaesthesia. Blood and brain tissues were collected promptly. Some of the brain tissues were frozen for further...
analysis, whereas the remaining brain tissues were fixed in 4% paraformaldehyde for hematoxylin-eosin (HE) or toluidine blue staining. The brain tissues were dehydrated in graded ethanol and embedded in paraffin. Then, paraffin sections were sliced and dewaxed with xylene. The slices were stained with HE to calculate the percentage of infarction and with toluidine blue to calculate the number of damaged neurons.

**Cytokines analysis**

The levels of S100 calcium-binding protein B (S100B), neuron-specific enolase (NSE), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), intercellular adhesion molecule-1 (ICAM-1), and cyclooxygenase 2 (COX-2) in serum were determined by ELISA (Elabscience Biotechnology Co., Ltd., Wuhan, China) following the manufacturers’ instructions. The levels of total antioxidative capacity (T-AOC), catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), oxidized glutathione (GSSG), and malondialdehyde (MDA) in brain tissues were measured using the respective kits (Elabscience Biotechnology Co., Ltd.).

**Quantitative real-time PCR**

Total RNA was extracted from brain tissues using TRIZol reagent (Aidlab Biotechnologies Co., Ltd., Beijing, China) and then used as a template for reverse transcription into cDNA using a reverse transcription kit (Vazyme Biotech Co., Ltd., Nanjing, China). Quantitative real-time PCR (qRT-PCR) was performed on ABI QuantStudio 6 Flex (Thermo Fisher Scientific, USA). The relative expression of each mRNA was normalized to β-actin and analyzed by the $2^{\Delta\Delta CT}$ method. The primers are listed in Table 1.

**Western blot**

Protein expression was determined by western blot, which was performed as previously described (Miao et al. 2015). Nrf2 expression was measured in the cytoplasmic and nuclear

| Table 1. Primers used for qRT-PCR. |
|-------------------------------|-------------------|
| Primer | Primer sequences |
| β-Actin | Forward 5′-CACGATGGAGGGCGGGACTACAT-3′ |
| | Reverse 5′-TAAAGACCTCTATGCAAACAGT-3′ |
| Nrf2 | Forward 5′-CCCATTGAGGGCTGTGAT-3′ |
| | Reverse 5′-TTGAGCCTCTAGTTAGGC-3′ |
| HO-1 | Forward 5′-GCATGCCAGGATTTGTC-3′ |
| | Reverse 5′-GGTTCGCTTTCGTCCTG-3′ |
| NQO1 | Forward 5′-GGCTGGAGGACATCCATC-3′ |
| | Reverse 5′-GCACGCCAGCTTTCCTG-3′ |
| Keap1 | Forward 5′-GGATGTTAACCGAACCCTC-3′ |
| | Reverse 5′-AACGGCACTGTTGACATAG-3′ |

Figure 2. Effects of Pue, Tan IIA and Pue-Tan IIA treatment on the infarct volume and neurological function in MCAO rats. (A) Representative TTC-stained brain sections. The white area represents the infarcted brain and the red stained area represents the normal brain. (B) Quantitative analysis of infarct volume. (C) Quantification results of the neurological function scores. **p < 0.01 vs. the sham group; *p < 0.05, **p < 0.01 vs. the MCAO group; *p < 0.05 vs. the Pue group.
Figure 3. Effects of Pue, Tan IIA, and Pue-Tan IIA treatment on brain histopathological changes. (A) Representative HE-stained brain sections in different groups. (B) Representative Nissl-stained brain sections in different groups. (C) Quantitative analysis of infarct volume by HE staining. (D) The number of damaged neurons by Nissl staining. **p < 0.01 vs. the sham group; *p < 0.05, ##p < 0.01 vs. the MCAO group.
fractions. All other proteins were determined using tissue homogenate. Protein concentration was quantified by the BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Proteins were separated by 10% SDS-PAGE and then transferred to the PVDF membrane. The membranes were incubated with primary antibodies against Nrf2, Keap1, HO-1, and NQO1 overnight at 4°C, respectively. β-Tubulin, lamin B, and β-actin were used as the corresponding internal controls. The membranes were rinsed and incubated with secondary antibodies for 2 h at room temperature. The blots were developed using enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China).

Statistical analysis
All data were expressed as mean ± standard deviation and analyzed using a one-way analysis of variance using GraphPad Prism 8.0.2 statistical software (GraphPad Software, Inc., La Jolla, San Diego, CA, USA). *p < 0.05 was considered statistically significant.

Results
Pue-Tan IIA alleviated ischaemic brain injury in MCAO rats
Infarct volume and neurological function were evaluated to determine the potential role of Pue-Tan IIA in IS (Figure 2). Except for the sham group, all the groups showed varying degrees of damage, especially the MCAO groups. In contrast with the MCAO group, the infarct volume and neurological function scores in the treatment groups were reduced, especially in the Pue-Tan IIA group (**p < 0.01). The scores of the Pue-Tan IIA group were significantly lower than those of the Pue group (*p < 0.05, #p < 0.01 vs. the MCAO group).

Histopathological changes were assessed by HE staining and Nissl staining, respectively (Figure 3). Almost no neuronal necrosis was observed in the sham group, whereas obvious infarcts were observed in the brain tissue of MCAO rats, with loose and disordered cell arrangement, enlarged intercellular spaces, pyknosis of the nucleus, hollow cytoplasm, and large portions of neuronal necrosis. After administration, the infarcts in each group were significantly reduced, which suggested that all treatments suppressed the damage to brain tissue to varying degrees. In contrast with the MCAO group, the infarct volume in the Pue-Tan IIA group was significantly reduced (**p < 0.01), and the number of damaged neurons was significantly reduced (#p < 0.01).

S-100β and NSE levels were measured to further determine the severity of brain injury (Said et al. 2021). As shown in Figure 4, S-100β and NSE levels in the MCAO group were significantly increased compared with the sham group (p < 0.01). Compared with the MCAO group, S-100β and NSE levels in the treatment groups were reduced, especially in the Pue-Tan IIA group (p < 0.01). These results indicated that Pue-Tan IIA could alleviate ischaemic brain injury.

Pue-Tan IIA inhibited oxidative stress and inflammation in MCAO rats
To evaluate the effects of Pue-Tan IIA on oxidative stress in MCAO rats, antioxidant parameters, such as T-AOC, CAT, SOD, GSH, GSSG, and MDA, were measured. The results were shown in Figure 5. Compared with the sham group, T-AOC, CAT, SOD, and GSH activities were significantly reduced (p < 0.01), and GSSG activity and MDA levels were significantly increased in the MCAO group (p < 0.01). Treatment in MCAO rats restored T-AOC, CAT, SOD, and GSH activities and reduced GSSG activity and MDA level. Pue-Tan IIA treatment significantly increased T-AOC, CAT, SOD, and GSH activities and significantly reduced GSSG activity and MDA level (p < 0.01).

TNF-α, IL-6, ICAM-1, and COX-2 levels in the MCAO group were significantly higher than those in the sham group (p < 0.01) (Figure 6). After administration, TNF-α, IL-6, ICAM-1, and COX-2 levels in MCAO rats were obviously reduced, especially in the Pue-Tan IIA group (p < 0.01). These results indicated that Pue-Tan IIA could inhibit oxidative stress and inflammation in MCAO rats.

Pue-Tan IIA activated Nrf2-ARE signalling pathway in MCAO rats
To determine the effect of Pue-Tan IIA on the Nrf2-ARE signalling pathway, the expressions of the relative proteins were investigated by qRT-PCR and western blots. As shown in Figure 7, the mRNA expression of Nrf2, HO-1, and NQO1 in MCAO rats
was significantly upregulated and increased more with treatment. Moreover, the mRNA expression levels of Nrf2 and HO-1 in the Pue-Tan IIA group were significantly higher than those in the Pue or Tan IIA group \((p < 0.05, \text{or} \ p < 0.01)\). Keap1 mRNA expression was significantly upregulated after MCAO \((p < 0.05)\) but downregulated with treatment.

Western blot results were shown in Figure 8. Compared with the sham group, the expression levels of Nrf2 in the cytoplasm
and nucleus were significantly increased in the MCAO group \( (p < 0.01, \text{ or } p < 0.05) \). After administration, Nrf2 expression in the cytoplasm was downregulated, whereas that in the nucleus was upregulated. The expressions of Keap1, HO-1, and NQO1 were significantly upregulated in the MCAO group compared with those in the sham group. In contrast with the MCAO group, the expression levels of HO-1 and NQO1 in the treatment groups were obviously increased, whereas Keap1 expression was obviously decreased. The expression levels of HO-1, NQO1, and Keap1 in Pue-Tan IIA group were significantly higher than those in the Pue group \( (p < 0.01) \). These results suggested that both Pue and Tan IIA could activate the Nrf2/ARE signalling pathway, and compared with either monotherapy, Pue-Tan IIA treatment enhanced the activation of this pathway.

**Discussion**

The global incidence of IS has been increasing in recent years, and yet, clinical treatment remains quite limited. Meanwhile, because of the narrow therapeutic window, a large proportion of patients still suffer severe adverse reactions after treatment, which seriously affects their quality of life (Wang et al. 2015; Snow 2016). Therefore, exploiting novel therapeutic approaches and medications remains highly imperative for IS. In this study, the combination of Pue and Tan IIA could significantly reduce the neurological function scores, infarct volume, and S-100β and NSE levels in the MCAO rats, indicating the significant ability of Pue-Tan IIA to alleviate ischaemic brain injury.

Increasing evidence has confirmed oxidative stress and inflammation play pivotal roles in the pathogenesis of ischaemic brain injury (Naderi et al. 2017; Zhang et al. 2019; Shaafi et al. 2021). After cerebral ischaemia, copious reactive oxygen species (ROS) are generated in brain tissue. The ensuing imbalance between ROS production and the ability of antioxidant systems (such as CAT, SOD, and GSH) to remove ROS results in excessive ROS accumulation that causes significant damage. ROS will interact with proteins, lipids, RNA, and DNA, leading to oxidative stress, inflammatory response, cell apoptosis, and ultimately, neuronal cell death (Jangholi et al. 2020; Tang et al. 2020; Wu et al. 2021). Besides the antioxidant systems, numerous cytokines, including TNF-α, IL-6, and COX-2, are released from the immune cells and secreted via brain cells (Kim et al. 2017; Yang et al. 2020). These cytokines can also enhance the expression of adhesion molecules (such as ICAM-1) in cerebrovascular endothelial cells to promote local inflammatory reactions (Wang et al. 2020). Therefore, inhibition of oxidative stress and the inflammatory response is an important intervention strategy in IS. In the

![Figure 6. Effects of Pue, Tan IIA, and Pue-Tan IIA treatment on the levels of inflammatory mediators in MCAO rats. (A) TNF-α; (B) IL-6; (C) ICAM-1; (D) COX-2.](image)

\(^{*}p < 0.01 \text{ vs. the sham group}; ^{*}p < 0.05, {^{##}p < 0.01} \text{ vs. the MCAO group.}\)
present study, T-AOC, CAT, SOD, and GSH activities were reduced, whereas GSSG, MDA, TNF-α, IL-6, ICAM-1, and COX-2 levels were increased in MCAO, indicating the induction of oxidative stress and inflammatory in IS. Consistent with previous studies (Cheng et al. 2016; Cai et al. 2017), both Pue and Tan IIA obviously reduced the extent of oxidative stress and inflammatory response by enhancing antioxidant activity, thereby improving the ability to scavenge free radicals, and reducing lipid peroxide production and the expressions of inflammatory factors.

Relevant studies consider the Nrf2/ARE signalling pathway as a key cellular defense mechanism, and its activation can alleviate ischaemic brain injury (Bellezza et al. 2018; Liu et al. 2019; Yu and Xiao 2021). Nrf2, a master endogenous antioxidant defense regulator, is an essential transcription factor that mediates ROS production and maintains redox homeostasis (Zhang et al. 2017; Yuan et al. 2021). Under normal circumstances, Nrf2 is mainly located in the cytoplasm. Under oxidative stress or inflammatory response, Nrf2 is released and translocated by Keap1 into the nucleus (Lu et al. 2021). Nrf2 binds to the ARE sequences and initiates downstream antioxidation and detoxification enzymes, such as HO-1, NQO1, SOD, CAT, and GSH (Sui et al. 2020). In the current study, the key protein expressions of the Nrf2/ARE signalling pathway were increased in the MCAO model, indicating that this pathway was activated against brain tissue damage caused by cerebral ischaemia. Treatment with Pue-Tan IIA significantly increased Nrf2 expression in the nucleus and decreased its expression in the cytoplasm, suggesting that Pue-Tan IIA promoted the nuclear transcription of Nrf2. Pue-Tan IIA significantly increased the expressions of HO-1 and NQO1 and decreased Keap1 expression. Meanwhile, the activities of SOD, CAT, and GSH increased with Pue-Tan IIA treatment. These results demonstrate that the neuroprotective effect of Pue-Tan IIA is mediated by the Nrf2/ARE signalling pathway.

Conclusions

The present study demonstrated that the combination of Pue and Tan IIA could alleviate ischaemic brain injury by inhibiting oxidative stress and inflammation in MCAO rats. The results also indicated that the Nrf2/ARE signalling pathway is involved in the protective mechanism of Pue-Tan IIA in IS. Furthermore, the neuroprotective effect of Pue-Tan IIA treatment is superior to Pue or Tan IIA monotherapy, emphasizing the synergistic effect of Pue and Tan IIA on alleviating ischaemic brain injury. These findings provided an experimental basis for the promising clinical application of Pue and Tan IIA combination therapy. However, this experiment is a preliminary study on Pue-Tan IIA.

Figure 7. Effects of Pue, Tan IIA, and Pue-Tan IIA treatment on the mRNA expressions of (A) Nrf2, (B) HO-1, (C) NQO1, and (D) Keap1 in MCAO rats. *p < 0.05, ##p < 0.01 vs. the sham group; #p < 0.05, ##p < 0.01 vs. the MCAO group; $p < 0.05, $$p < 0.01 vs. the Pue group; &p < 0.05, &&p < 0.01 vs. the Tan IIA group.
Figure 8. Effects of Pue, Tan IIA, and Pue-Tan IIA treatment on the protein expression of Nrf2, HO-1, NQO1, and Keap1 in MCAO rats. (A) Representative western blot and quantitative analysis of Nrf2 in cytoplasm in different groups. β-Tubulin was used as an internal control. (B) Representative western blot and quantitative analysis of Nrf2 in the nucleus in different groups. Lamin B was used as an internal control. (C) Representative western blot and quantitative analysis of HO-1, NQO1, and Keap1 in different groups. β-Actin was used as an internal control. *$p < 0.05$, **$p < 0.01$ vs. the sham group; #*$p < 0.05$, #*$p < 0.01$ vs. the MCAO group; &&*$p < 0.01$ vs. the Pue group.
treatment in IS, and more in-depth research into the pharmacologic mechanisms is required.

Disclosure statement

The authors report there are no competing interests to declare.

Funding

This work was supported by the Fundamental Research Funds for the Central Public Welfare Research Institutes [YZ-1810].

References

Bai YT, Lv Y. 2014. Comparative study on the efficacy of puerarin combined with tanshinone on patients with affective disorder after first acute cerebral infarction. Med J Nation Defending Forces Northwest China. 35:1022–374.

Bellezza I, Giambanco I, Minelli A, Donato R. 2018. Nrf2-Keap1 signaling in oxidative and reductive stress. Biochim Biophys Acta Mol Cell Res. 1865(5):721–733.

Cai M, Guo YX, Wang SQ, Wei HD, Sun SS, Zhao GC, Dong HL. 2017. Comparative study on the efficacy of puerarin combined treatment in IS, and more in-depth research into the pharmaco-

logic mechanisms is required.

Chen YL, Wu XM, Yu SS, Fauzee NJS, Wu JX, Li L, Zhao J, Zhao Y. 2012. Neuroprotective capabilities of tanshinone IIA against cerebral ischemia/reperfusion injury via anti-apoptotic pathway in rats. Biol Pharm Bull. 35(2):164–170.

Cheng Y, Leng W, Zhang JS. 2016. Protective effect of puerarin against oxidative stress injury of neural cells and related mechanisms. Med Sci Monit. 22:1244–1249.

Fisher M. 2011. New approaches to neuroprotective drug development. Stroke. 42(1 Suppl):S24–S27.

Gao S, Li LY, Li L, Ni JY, Guo R, Mao JY, Fan GW. 2019. Effects of the combination of tanshinone IIA and puerarin on cardiac function and inflammatory response in myocardial ischemia mice. J Mol Cell Cardiol. 137:59–70.

Gorelick PB. 2019. The global burden of stroke: persistent and disabling. Lancet Neurol. 18(5):417–418.

Jangholi E, Sharifi ZN, Hoseinian M, Zarrindast MR, Rahimi HR, Mowla A, Aryan H, Javidi MA, Parsa Y, Ghaffarpasand F, et al. 2020. Verapamil inhibits mitochondria-induced reactive oxygen species and dependent apoptosis pathways in cerebral transient global ischemia/reperfusion. Oxid Med Cell Longev. 2020:5872645.

Kim E, Kim HC, Lee S, Ryu HG, Park YH, Kim JH, Lim YJ, Park HP. 2017. Dexametomidine confers neuroprotection against transient global cerebral ischemia/reperfusion injury in rats by inhibiting inflammation through inactivation of the TLR-4/NF-κB pathway. Neurosci Lett. 649:20–27.

Kong H, Zhang GL, Cheng J, Shi RF, Zhang ML, Gao P, Zhao Y, Qu HH, Wang QG. 2019. Distribution kinetics of puerarin in rat hippocampus after acute local cerebral ischemia. J Pharm Biomed Anal. 164:196–201.

Li YH, Wang FY, Feng CQ, Yang XF. 2015. Studies on the active constituents in radix Salviae miltiorrhizae and their protective effects on cerebral ischemia reperfusion injury and its mechanism. Pharmaco-nig. Mag. 11(41):69–73.

Liu L, Locascio LM, Doré S. 2019. Critical role of Nrf2 in experimental ischemic stroke. Front Pharmacol. 10:153.

Liu B, Tan Y, Wang DR, Liu M. 2016. Puerarin for ischaemic stroke. Cochrane Database Syst Rev. (2):CD004955.

Longa EZ, Weinstein PR, Carlson S, Cummins R. 1989. Reversible middle cerebral artery occlusion without cranectomy in rats. Stroke. 20(1):84–91.

Lu JQ, Gu LL, Li Q, Wu NZ, Li HX, Zhang XY. 2021. Andrographolide eme-

lates maltool aluminium-induced neurotoxicity via regulating p62-medi-

ated Keap1-Nrf2 pathways in PC12 cells. Pharmacol Biol. 59(1):232–241.

Lv JS. 2016. Shijinmo Duiyao. Beijing: People’s Military Medical Press; p. 154–155.

Miao Q, Zhao YY, Miao PP, Chen N, Yan XH, Guo CE, Chen HY, Zhang YJ. 2015. Proteomics approaches to analyze protein profiling related with ADME/Tox in rat treated with Scutellariae radix and Capitidis rhizoma as well as their compatibility. J Ethnopharmacol. 173:241–250.

O'Shea Y, Sobotkova M, Parvardeh S, Janzani TM. 2017. Neuroprotective effect of minocycline on cognitive impairments induced by transient cere-

bral ischemia/reperfusion through its anti-inflammatory and anti-oxidant properties in male rat. Brain Res Bull. 131:207–213.

Paid MF, Islam AA, Massi, MN, Prihantono. 2021. Effect of erythropoietin administration on the expression of brain-derived neurotrophic factor, stromal cell-derived Factor-1, and neuron-specific enolase in traumatic brain injury: a literature review. Ann Med Surg. 62:26666.

Shaafi S, Hadisi F, Mahmoudinezhad M, Razmi H, Nejadghaderi SA, Khalili M. 2021. The significance of the oxidative stress markers in the one-year prognosis of patients with acute ischemic stroke: a case-control study. BMC Neurolog. 21(1):258.

Snow SJ. 2016. Stroke and t-PA – triggering new paradigms of care. N Engl J Med. 374(9):809–811.

Sui YB, Zhang KK, Ren YK, Liu L, Liu Y. 2020. The role of Nrf2 in astragalo-

side IV-mediated antioxidative protection on heart failure. Pharmacol. 58(1):1192–1198.

Sun XX, Miao Q, Wang RH, Liu LM. 2020. Research advances on chemical constituents, pharmacological effects and clinical applications of Salvia miltiorrhiza-Puerariae lobatae radix herb pair. J Basic Chin Med. 22:430–4313.

Tang CL, Hu YD, Lyu HY, Gao J, Jiang JZ, Qin XD, Wu YB, Wang JW, Chai XQ. 2020. Neuroprotective effects of 1-O-hexyl-2,3,5-trimethylhydro-

quinone on ischemia/reperfusion-induced neuronal injury by activating the Nrf2/HO-1 pathway. J Cell Mol Med. 24(18):10468–10477.

Wang W, Li MC, Chen QX, Wang J. 2015. Hemorrhagic transformation after tissue plasminogen activator reperfusion therapy for ischemic stroke: mechanisms, models, and biomarkers. Mol Neurobiol. 52(3):1572–1579.

Wang YL, Xiao GX, He S, Liu YX, Zha L, Yang YX, Yang QY, Organ G, Feng YX, Wang XY, et al. 2020. Protection against acute cerebral ische-

mia/reperfusion injury by QShenYiQi via neuroinflammatory network modulation. Biomed Pharmacother. 125:109945.

Wu YT, Xie LP, Hua Y, Xu HL, Chen GH, Han XT, Tan ZB, Fan HJ, Chen HM, Li J, et al. 2021. Tanshinone I inhibits oxidative stress-induced cardia-

myocyte injury by modulating Nrf2 signaling. Front Pharmacol. 12:644116.

Wu SY, Wang E, Chen F, Xiao JB, Wang MF. 2021. Neuroprotective phyto-

chemicals in experimental ischemic stroke: mechanisms and potential clinical applications. Oxid Med Cell Longev. 2021:6687386.

Xu XH, Wang JB, Zhang H, Tian GQ, Liu YQ. 2016. Puerarin reduces apo-

tosis in rat hippocampal neurons culture in high glucose medium by mod-

ulating the p38 mitogen activated protein kinase and c-Jun N-terminal kinase signaling pathways. J Tradit Chin Med. 36:78–84.

Yang TS, Feng CW, Wang DY, Qu YY, Yang Y, Wang YL, Sun ZR. 2020. Neuroprotective and anti-inflammatory effect of Tangeretin against cere-

bral ischemia-reperfusion injury in rats. Inflammation. 43(6):2332–2343.

Yang X, Xiong W, Feng J. 2014. Treatment with tanshinone IIA suppresses the suppression of the blood-brain barrier and reduces expression of adhesion molecules and chemokines in experimental autoimmune encephalomyeli-

tis. Eur J Pharmocol. 771:18–28.

Yu C, Xiao JH. 2021. The Keap1-Nrf2 system: a mediator between oxidative stress and aging. Oxid Med Cell Longev. 2021:6635460.

Yuan H, Xu Y, Luo Y, Wang NX, Xiao JH. 2021. Role of Nrf2 in cell senes-
cence regulation. Mol Cell Biochem. 476(1):247–259.

Zhang L, Li QS, Huang Y. 2020. Study on utilization safety and rationality of sodium tanshinone II_A sulfonic injection based on “real world”. China Pharm. 31:217–220.

Zheng H, Wang JH, Wang YL, Gao C, Gu YT, Huang J, Wang JH, Zhang Z. 2019. Salvinolic Acid A protects the kidney against oxidative stress by activating the Akt/GSK-3β/Nrf2 signaling pathway and inhibiting the NF-

κB signaling pathway in 5/6 nephrectomized rats. Oxid Med Cell Longev. 2018:2835354.

Zhang RR, Xu MX, Wang Y, Xie F, Zhang G, Qin XY. 2017. Nrf2-a promis-

ing therapeutic target for defending against oxidative stress in stroke. Mol Neurobiol. 54(8):6006–6017.

Zhang QH, Li XL, Mei ZG, Song L, Mei QX, Wang JF, Tan LJ, Yang SB, Feng ZT. 2017. Efficacy and safety of puerarin injection in curing acute ischemic stroke: a meta-analysis of randomized controlled trials. Medicine. 96(1):e5803.

Zhou MG, Wang HD, Zeng XY, Yin P, Zhu J, Chen WQ, Li XH, Wang LJ, Wang LM, Liu YN, et al. 2019. Mortality, morbidity, and risk factors in China and its provinces, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 394(10245):1145–1158.