International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2020.214.222

Research Article
Tao-Hong Si-Wu Decoction Alleviates Cerebral Ischemic Damage in Rats by Improving Anti-oxidant and Inhibiting Apoptosis Pathway

[1,2]Mengmeng Wang, [1,2]Feilong Wang, [1,2,3]Daiyin Peng, [1,2]Xianchun Duan, [1,2]Weidong Chen, [1,2]Fan Xu and [1,2]Lan Han

[1]School of Pharmacy, Anhui University of Chinese Medicine, 230012 Hefei Anhui, China
[2]Anhui Province Key Laboratory of Chinese Medicinal Formula, 230012 Hefei Anhui, China
[3]Key Laboratory of Xin'an Medicine, Ministry of Education, Anhui University of Chinese Medicine, 230012 Hefei Anhui, China

Abstract

Background and Objective: Tao-Hong-Si-Wu decoction (THSWD) as a classical prescription in traditional Chinese medicine, has been developed to treat blood stasis syndrome for hundreds of years. THSWD is highly effective in treating focal cerebral ischemia. The purpose of this study was to evaluate the anti-oxidative and anti-apoptotic activity of THSWD on middle cerebral artery occlusion (MCAO) in rats. **Materials and Methods:** The rats were randomly divided into 6 groups: sham group, model group, nimodipine group (0.02 g/kg/day) and 3 treatment groups by THSWD at 4.5, 9, 18 g/kg/day. After a consecutive oral administration for 7 days. The ischemic injury was assessed by mensurating brain infarct volume, brain water content and observing morphologic characteristics of ischemic cerebral cortex. Also, the levels of nitric oxide (NO), malondialdehyde (MDA) and catalase (CAT), the activities of total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px) were detected to investigate the antioxidant mechanisms. Western Blotting was used to shed a light on the expression of B cell lymphoma 2 (Bcl-2) and Bax to clarify the anti-apoptotic mechanisms. **Results:** THSWD significantly reduced infarction volume and brain water content. The positive nuclei of the THSWD treatment group increased significantly and were neatly arranged. Nissl stained neurons and intact cells were found in THSWD group, indicating that MCAO was recovered by THSWD. THSWD could increase the activities of SOD, CAT, GSH-Px and decrease MDA, NO levels in the cerebral cortex. Besides, THSWD could reduce apoptosis damage of brain tissues by up-regulating Bcl-2 level, down-regulating Bax level and increasing Bcl-2/Bax ratio. **Conclusion:** These results suggest that THSWD ameliorate neurological dysfunction of focal cerebral ischemia injury, which might be related to the modulation of multiple anti-oxidant and anti-apoptotic pathways.

Key words: Focal cerebral ischemia/reperfusion, Tao-Hong-Si-Wu decoction, anti-oxidation, anti-apoptotic

Citation: Mengmeng Wang, Feilong Wang, Daiyin Peng, Xianchun Duan, Weidong Chen, Fan Xu and Lan Han, 2020. Tao-Hong Si-Wu decoction alleviates cerebral ischemic damage in rats by improving anti-oxidant and inhibiting apoptosis pathway. Int. J. Pharmacol., 16: 214-222.

Corresponding Author: Lan Han, School of Pharmacy, Anhui University of Chinese Medicine, 230012 Hefei Anhui, China

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stroke is the third leading cause of mortality worldwide and with a permanent disability, out of which about 80% are ischemic strokes[1,2]. There is growing evidence shows that oxidative stress is involved in the pathogenesis of ischemic stroke. And it is considered to be an early and the most important pathological factor of tissue injury after ischemic stroke, which has been shown accompanied with worse functional outcome[3]. Oxidative stress and production of reactive oxygen species (ROS) are implicated in the initiation of apoptosis processes[4]. Although the mechanisms of apoptosis during ischemic strokes remain unclear, many studies showed that the balance between pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 are a critical determinant of regulating apoptosis[5].

Traditional Chinese Medicine gains more and more attention nowadays, natural products have been clinically used for the treatment of stroke owing to their brilliant efficacy in neuroprotective and neurorestorative actions. Tao-Hong Si-Wu decoction (THSWD) is a famous traditional Chinese medicine formula, which was first appeared in Yizong jinjian by Wu Qian and widely used to treat blood stasis syndrome in China for hundreds of years. This formula contains six commonly used herbs, including *Prunus* Semen, *Carthami* Flos, *Angelicae sinensis* Radix, *Chuanxiong rhizoma*, *Paeoniae Alba* Radix and *Rehmanniae preparata* Radix. To treat ischemic stroke, traditional Chinese medicine practitioners prescribe herbs that can dilate the blood vessels and promote blood circulation. Researchers had shown that hydroxysafflor yellow A, ferulic acid and ligustilide played a therapeutic role in cerebral ischemia/reperfusion rats[6-8]. Meanwhile, the three compounds can be detected in serum after treatment THSWD[9].

Previous research indicated that THSWD possessed potent neuroprotective activity against brain damage in rat with cerebral ischemic injury[10-12]. However, whether THSWD has a neuroprotective effect against apoptosis and oxidant in focal cerebral ischemia/reperfusion (I/R) injury remains unclear. Therefore, the present study endeavors to investigate the potential neuroprotective effects of THSWD on cerebral ischemia/reperfusion injury and elucidate the underlying therapeutic mechanism.

MATERIALS AND METHODS

Composition and preparation of THSWD: THSWD consist of 6 medicinal plants as shown in Table 1. *Prunus* Semen (Taoren in Chinese, TR), *Carthami* Flos (Honghua in Chinese, HH), *Angelicae sinensis* Radix (Danggui in Chinese, DG), *Chuanxiong* Rhizoma (*Chuanxiong* in Chinese, CX), *Paeoniae Alba* Radix (Baishao in Chinese, BS) and *Rehmanniae preparata* Radix (Shudihuang in Chinese, SDH) (batch number: NO:151124, NO:151230, NO:151216, NO:151221, NO:151124, NO:151222) were purchased from Bozhou Yonggang Pieces Factory Co. Ltd. (Anhui, China) and identified by Prof. Dequn Wang (Professor of Pharmacology, Anhui University of Chinese Medicine, China). The air-dried herbs were immersed in a total volume of 10 times (V/W) 75% ethanol for 6 h and then boiled for 2 h and the decocted liquid was taken out. The residue was refluxed again for 2 h with eight times using 75% ethanol (V/W). After that, the filtrate was collected and concentrated to 1.8 g mL^{-1}. To ensure the quality and stability of the THSWD, we used Ultra Performance Liquid Chromatography (UPLC) to identify the components[13].

Table 1: Herbals of Tao Hong Si-Wu decoction ingredient

Ingredient	Part used	Weight (g)
Shu Di Huang *(Rehmannia glutinosa* Libosch.)	Radix	12
Dang Gui (*Angelica sinensis* (Oliv.) Diels)	Radix	9
Bai Shao (*Paeonia lactiflora* Pall.)	Radix	9
Chuan Xiong (*Ligusticum chuanxiong* Hort.)	Rhizoma	6
Tao Ren (*Prunus persica* (L.) Batsch)	Semen	9
Hong Hua (*Carthamus tinctorius* L.)	Flos	6

Animals and experimental protocol: Sprague-Dawley male rats (250±20 g) were offered by the Laboratory Animal Center of Anhui Medical University (Certificate NO.SCXK2011-002), conform to the principles of laboratory animal health guide for care and the experimental study was approved by the Ethics Committee of Anhui University of Chinese Medicine. This animal study was at the School of Pharmacy of Anhui University of Chinese Medicine, Hefei, China from 2nd-28th November, 2016.

Focal cerebral ischemia/reperfusion model: The middle cerebral artery occlusion (MCAO) surgery was performed[14]. For anesthetized rats, a short incision was made to expose and isolate the right common carotid artery (CCA). A diameter of 0.285 mm silicon-coated nylon was impelled gently from external carotid artery (ECA) via bifurcation to internal carotid artery (ICA) to block MCA, nearly 18-20 mm from bifurcation to MCA. After occlusion for 2 h, the filament was removed to allow reperfusion. Rats in the sham group underwent the same surgical procedures without insertion of the filament.

Drug administration protocol: Following that, rats were randomly divided into 6 groups: sham group, model group,

3 different doses for THSWD groups and nimodipine group. The administered doses of THSWD were 4.5, 9 and 18 g/kg/day and nimodipine was 0.02 g/kg/day, respectively, once a day for 7 consecutive days after modeling. Rats in sham group and model group were administered with normal saline in the same volume.

Measurement of infarct volume: The rats were sacrificed and brain was immediately removed, sliced into 2 mm thick coronal sections. The sections were immediately stained with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) for 30 min at 37°C in the dark. The infarct volume was calculated using Image-pro plus (IPP 6.0, Media Cybernetics, MD, USA) software as previously described by Lu *et al.*[15].

Measurement of brain water content: Seven days after MCAO, brain water content was determined to utilize the standard wet-dry method[16]. The brain water content (BW) was then calculated as follows:

$$\text{In brain water content should be BW (\%)} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weigh}} \times 100\ (\%)$$

Pathological examination of brain tissue: The brains were fixed in 4% paraformaldehyde solution for over 24 h, processed routinely for paraffin embedding. Brain sections stained (3 μm-thick) were stained with hematoxylin-eosin (H-E) to examine the pathological change of the cerebral ischemic penumbra by light microscopy (×200). To detect morphological changes in neurons, the slices (5 μm-thick) were dewaxed to water and subjected to Nissl staining using cresyl violet stain.

Measurement of oxidative stress-related biological parameters: The penumbra of ischemic cortex were homogenized in 10% (w/v) physiological saline (n = 6). Followed by centrifugation at 4°C, the supernatant was examined as soon as possible to determine the levels of NO and MDA and T-SOD, GSH-Px and CAT according to the manufacturer's instructions[17,18]. The kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Western blotting analysis: The penumbra of ischemic cortex was isolated and homogenized in lysis buffer on ice. The homogenate was centrifuged at 12000 g for 10 min at 4°C and the total supernatant protein was measured by BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Then, equal amounts of protein lysates were separated by SDS-PAGE and transferred to nitrocellulose filter (NC) membranes. The membranes were blocked using 5% skim milk for 2 h and then incubated with the following primary antibodies: Bax (1:1000, Abcam, USA), Bcl-2 (1:1000, Abcam, USA), β-actin(1:1000, Zhongshan Golden Bridge Biotechnology Co., China) overnight at 4°C. Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG at room temperature for 1.5 h. The blot was then visualized by ECL kit (Amer cataway, NJ, USA). Finally, Image J analysis software (Image J, NIH) was used to quantitative analysis.

Statistical analysis: The data were analyzed with SPSS 20.0 software and continuous variables were described as the Mean±SEM. Multigroup mean comparisons were performed using a one-way ANOVA. The $p<0.05$ was considered to indicate a significant difference.

RESULTS

Effect of THSWD on MCAO-induced cerebral ischemic Injury: Ischemic tissue was not stained red. No infarction was seen in rats of sham group (Fig. 1a). A tiny infarct area was observed in rats of THSWD group. Administration of nimodipine and THSWD (4.5, 9 and 18 g kg^{-1}) (Fig. 1b) could significantly decrease the infarct volume in rats with cerebral ischemic injury ($p<0.01$ or $p<0.05$). In each group, brain edema of the ischemic hemisphere was shown in Fig. 1c. The nimodipine and THSWD groups could ameliorate brain edema in ischemic hemisphere. Compared with the model group, there was a significant reduction in brain water content in THSWD (4.5, 9 and 18 g kg^{-1}) group ($p<0.01$ or $p<0.05$).

Amelioration of histopathological alteration: As shown in Fig. 2, histological changes in the neurons of the cortical region were obtained by HE staining in all groups. The sham group showed intact neurons, abundant cytoplasm with no inflammatory cells infiltrated, whereas the MCAO group exhibited more disordered neurons, neuronal degeneration and necrosis as well as neuron shrunken neurons nuclear, swelled degeneration in glial cells. The nimodipine and THSWD (4.5, 9 and 18 g kg^{-1}) group markedly relieved the abnormalities caused by MCAO. Moreover as shown in Fig. 3, Nissl staining showed the decreased number of neurons in the penumbra of ischemic cortex were found after MCAO surgery and most cells were shrunk with enlarging intercellular space. Compared with the model group, more Nissl stained neurons and intact cells were found in THSWD (4.5, 9 and 18 g kg^{-1}) group, indicating that MCAO was recovered by THSWD.

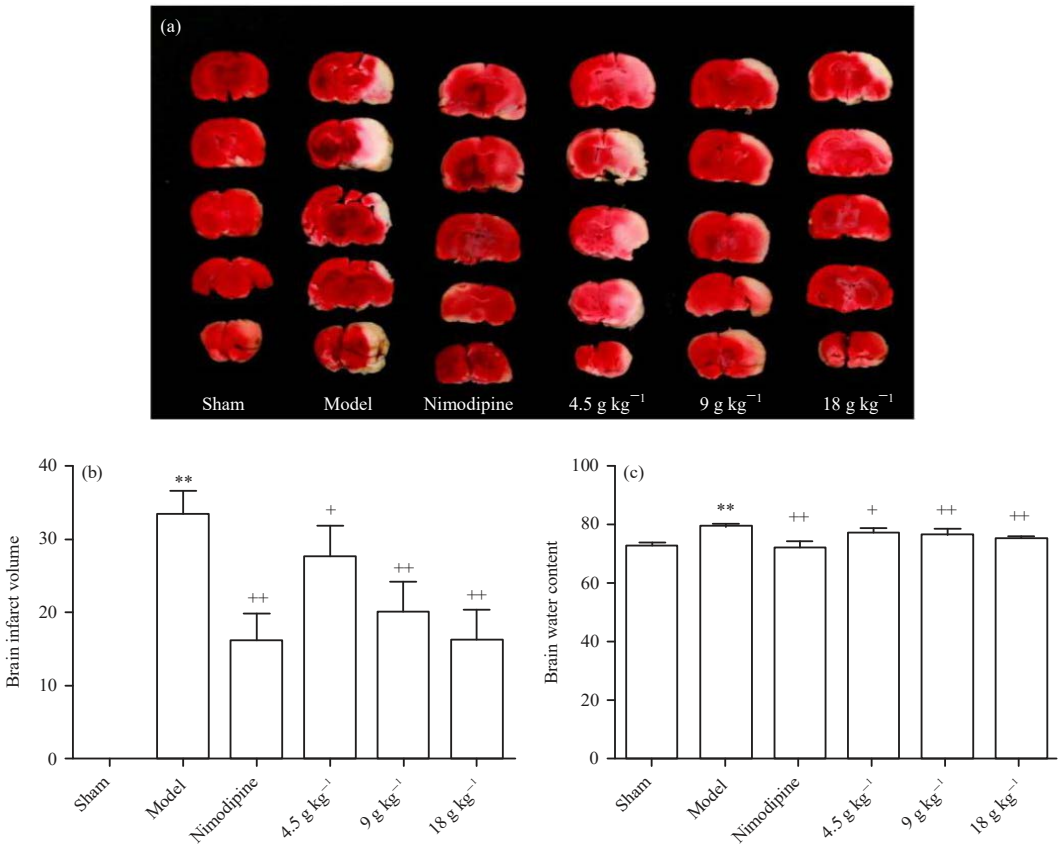

Fig. 1(a-c): Effect of THSWD on cerebral infarction and the water content of brain, (a) After the successive oral administration for 7 days, representative coronal sections stained with 2% TTC, (b) Quantitative analysis of the infarct volumes and (c) Quantitative analysis of the brain water content

Bars represent the Mean±SEM for each group (n = 6), **p<0.01 vs. sham, $^{+}$p<0.05, $^{++}$p<0.01 vs. model

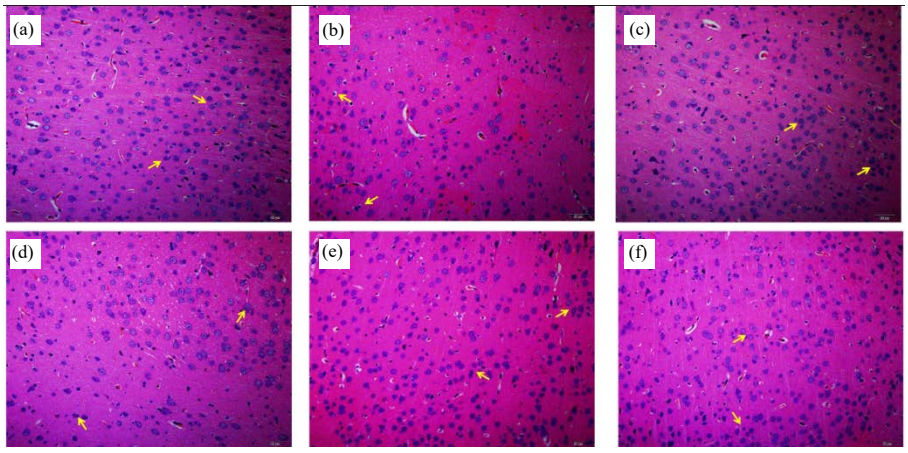

Fig. 2(a-f): Effects of THSWD treatment on the postischemic changes by HE staining (×200), (a) Sham group with normal neurons, (b) Model group exhibited the physiological abnormality, (c) Nimodipine group as positive control, showed the recovering neurons and (d) THSWD (4.5 g kg^{-1}), (e) THSWD (9 g kg^{-1}) and (f) THSWD (18 g kg^{-1}) group were administered to MCAO rats, respectively

Neurons were recovered at different levels

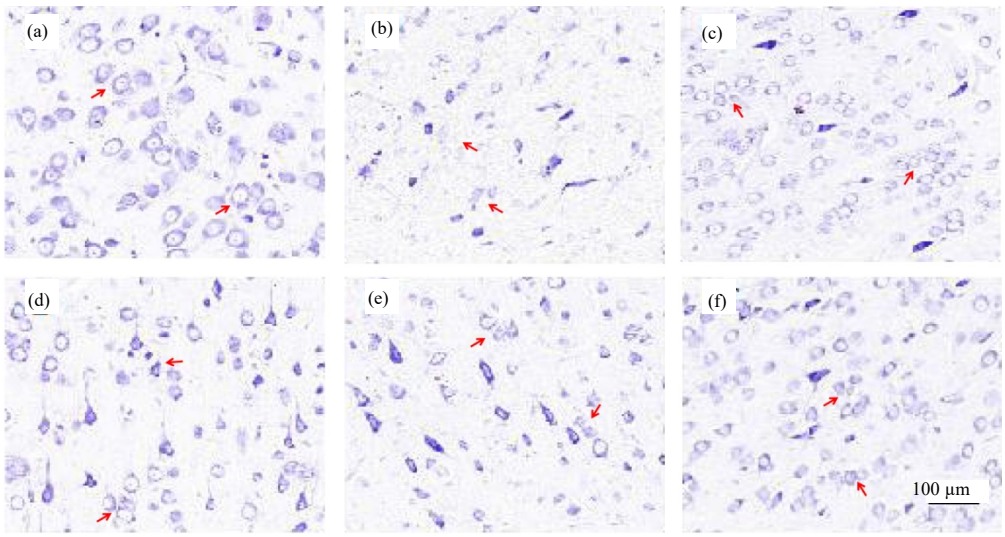

Fig. 3(a-f): Nissl staining of the cerebral ischemic penumbra after ischemia/reperfusion injury, (a) Sham group, (b) Model group, (c) Nimodipine group, (d) THSWD (4.5 g kg^{-1}) group, (e) THSWD (9 g kg^{-1}) group and (f) THSWD (18 g kg^{-1}) group

Effects of THSWD on the levels of NO, MDA and the activities of T-SOD, CAT, GSH-Px: As shown in Fig. 4, the levels of NO and MDA were significantly increased in model group compared with the sham group ($p<0.01$) and a dose dependent decrease was shown in THSWD group compared with the model group. Whereas a significant decrease in the activity of T-SOD, CAT and GSH-Px was found in model group compared with sham group ($p<0.01$). Furthermore, the activities of SOD, CAT, GSH-Px significantly increased in the nimodipine and THSWD group ($p<0.01$ or $p<0.05$).

Effects of THSWD on expressions of Bcl-2 and Bax: As shown in Fig. 5, to confirm the levels of Bcl-2 and Bax proteins were determined by western blot analysis with β-actin as an internal standard. Western blot results revealed a significant increase in the protein level of Bax and significantly decrease in Bcl-2 protein expression in model group ($p<0.01$), which indicates higher apoptosis existed in the cerebral cortex in the model group. The results of western blot also proved THSWD (9 and 18 g kg^{-1}) down-regulated expression of Bax and up-regulated expression of Bcl-2. Furthermore, THSWD (9 and 18 g kg^{-1}) treatment significantly increased Bcl-2/Bax ratio ($p<0.01$ or $p<0.05$).

DISCUSSION

MCAO is a widely accepted model of cerebral ischemia[14]. In the present study, we utilized the MCAO model to study the mechanism of THSWD against cerebral ischemia/reperfusion in rats. The brain injury of MCAO-induced rats were confirmed by increasing neurological dysfunction, brain edema and infarct volume[19]. The above results showed that compared with the model group, the cerebral infarction volume and brain water content decreased in the THSWD group. Moreover, the number of positive nuclei and brain Nissl bodies increased significantly. These results demonstrated that the neuroprotective ability of THSWD against ischemic stroke through anti-oxidant and anti-apoptotic mechanisms.

After the occurrence of stroke, the mitochondria of cells were damaged due to ischemia and hypoxia and the oxidative stress injury was caused by excessive pathological ROS. Of course, ROS could aggravated mitochondrial damage, led to energy metabolism disorders, inflammation and induced cell death[20,21]. Oxidative stress and apoptosis are the major causes of cerebral injury in ischemic stroke. Ischemic stroke hinders the balance between the formation and transformation of ROS[22,23], resulting in the accumulation of ROS. Then, neurotoxic peroxynitrite is formed by reacting with excessive endogenous NO, which triggered by cerebral ischemia/reperfusion[24]. MDA is a major biomarker of lipid peroxidation and indicates the extent of cell damage, which can also indirectly reflect the ability of the free radicals scavenging[25]. The key antioxidant enzymes, including SOD, CAT and GSH-Px, provide a defense system against oxidative stress in response to acute ischemia. SOD can scavenge ROS leading to protect the brain from ischemic injury[26,27]. It converts superoxide to peroxide ($2O_2^- + 2H \rightarrow H_2O_2 + O_2$), which is split to oxygen and water by CAT and GSH-Px. There was a

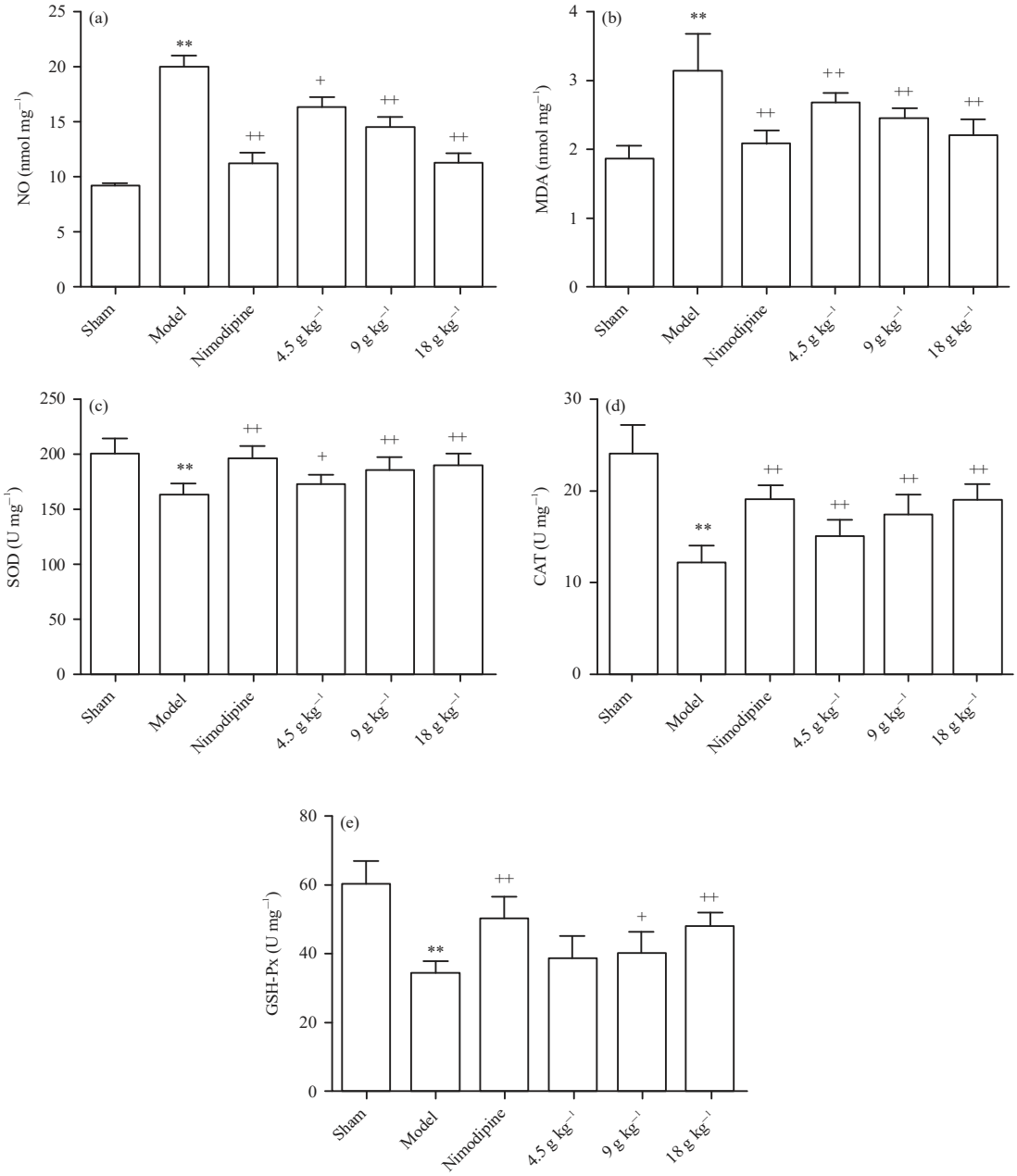

Fig. 4(a-e): Effects of THSWD on the levels of NO, MDA and the activities of T-SOD, CAT, GSH-Px in the brains of MCAO-induced rats, (a) Effects of treatment with THSWD on the contents of NO, (b) MDA, (c) T-SOD, (d) CAT and (e) GSH-Px
Bars represent the Mean±SEM for each group (n = 6), **p<0.01 vs. sham, $^{+}$p<0.05, $^{++}$p<0.01 vs. model

significantly increase in NO and MDA levels with the reduction in SOD levels in MCAO rats indicating ROS-antioxidant enzyme is an imbalance, which is consistent with previous report by Chauhan *et al.*[28] and Gupta *et al.*[29].

However, the present results showed that THSWD significantly reduced NO, MDA levels and increased SOD, CAT and GSH-Px activities. These results demonstrated that THSWD could protect the neurons from oxidative damage by eliminating free radical and reducing lipid peroxidation. Namely, THSWD was regarded as an antioxidant to play a fundamental role against cerebral ischemia/reperfusion through the attenuation of oxidative stress.

Cerebral ischemia/reperfusion can induce both necrotic and apoptotic cell death[30]. The Bcl-2 family plays an important role in the regulation of apoptotic cell death, including both positive and negative regulation of the apoptotic pathway.

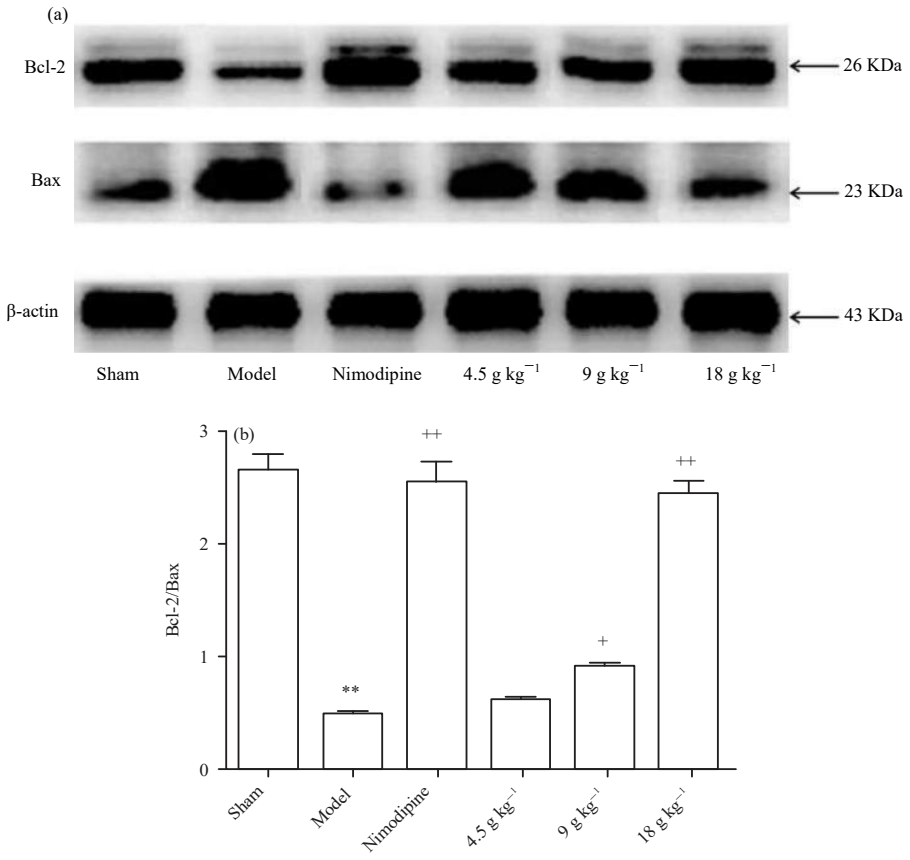

Fig. 5(a-b): Effects of THSWD on the expressions of Bcl-2 and Bax after cerebral ischemia in rats and (b) Changes in Bcl-2/Bax ratios

Bars represent the Mean±SEM for each group (n = 3), **p<0.01 vs. sham, $^{+}$p<0.05, $^{++}$p<0.01 vs. model

Bax and Bid are the pro-apoptotic members of this family, which promote apoptosis by transferring from the cytosol to the outer mitochondrial membrane, thereby leading to caspase activation[31-33]. In contrast, the Bcl-2 is the anti-apoptotic member of this family, which prevent cells from undergoing apoptosis caused by various stimuli. The Bcl-2 family maintains mitochondrial stabilization by regulating the Bcl-2/Bax balance[34]. Our study showed that THSWD could up-regulate Bcl-2 protein, down-regulated Bax and facilitated the ratio of Bcl-2/Bax, which was consistent with the previous studies by Han *et al.*[11]. It deduced that THSWD inhibited cerebral apoptosis by stabilizing the mitochondria and then blocked the release of pro-apoptotic inducing molecules. Our results demonstrated that THSWD inhibited cerebral ischemia-induced apoptosis, contributing to the neuroprotective effects. Namely, studies have shown that THSWD inhibits cerebral apoptosis, thereby preventing the transition into infarct and reducing overall infarct volume.

CONCLUSION

In conclusion, this study demonstrated that THSWD exerts neuroprotective effects against MCAO-induced injury. THSWD treatment after stroke could decrease the infarct volume, neurological scores and brain edema. These neuroprotective effects of THSWD likely occur to multiple mechanisms in ischemic stroke, including protecting the neurons from oxidative damage and apoptosis. Above all, THSWD could be used as a therapeutic agent with high promise for treating the ischemic stroke in the future clinic.

SIGNIFICANCE STATEMENT

The results showed that THSWD could play an important role in cerebral stroke by anti-oxidative stress injury and inhibition of apoptosis. The results of this experiment will be useful for finding alternative drugs for the treatment of

cerebral stroke and could be recommended along with common standard cerebral stroke treatment regimen to treat stroke patients.

ACKNOWLEDGMENT

This study was supported by the National Natural Science Foundation of China (grant no. 81473387 and 81503291) and the Key Research and Development Plan of Anhui Province (grant no. 1704a0802141).

REFERENCES

1. Doyle, K.P., R.P. Simon and M.P. Stenzel-Poore, 2008. Mechanisms of ischemic brain damage. Neuropharmacology, 55: 310-318.
2. Hong, K.S. and J.L. Saver, 2009. Quantifying the value of stroke disability outcomes: WHO global burden of disease project disability weights for each level of the modified Rankin Scale. Stroke, 40: 3828-3833.
3. Loh, K.P., J. Qi, B.K.H. Tan, X.H. Liu, B.G. Wei and Y.Z. Zhu, 2010. Leonurine protects middle cerebral artery occluded rats through antioxidant effect and regulation of mitochondrial function. Stroke, 41: 2661-2668.
4. Greenlund, L.J., T.L. Deckwerth and E.M. Johnson Jr., 1995. Superoxide dismutase delays neuronal apoptosis: A role for reactive oxygen species in programmed neuronal death. Neuron, 14: 303-315.
5. Wattanapitayakul, S.K. and J.A. Bauer, 2001. Oxidative pathways in cardiovascular disease: Roles, mechanisms and therapeutic implications. Pharmacol. Therapeut., 89: 187-206.
6. Lv, Y., Y. Qian, L. Fu, X. Chen, H. Zhong and X. Wei, 2015. Hydroxysafflor yellow A exerts neuroprotective effects in cerebral ischemia reperfusion-injured mice by suppressing the innate immune TLR4-inducing pathway. Eur. J. Pharmacol., 769: 324-332.
7. Cheng, C.Y., N.Y. Tang, S.T. Kao and C.L. Hsieh, 2016. Ferulic acid administered at various time points protects against cerebral infarction by activating p38 MAPK/p90RSK/CREB/Bcl-2 anti-apoptotic signaling in the subacute phase of cerebral ischemia-reperfusion injury in rats. PLoS ONE, Vol. 11, No. 5. 10.1371/journal.pone.0155748.
8. Kuang, X., L.F. Wang, L. Yu, Y.J. Li and Y.N. Wang *et al*., 2014. Ligustilide ameliorates neuroinflammation and brain injury in focal cerebral ischemia/reperfusion rats: Involvement of inhibition of TLR4/peroxiredoxin 6 signaling. Free Radic. Biol. Med., 71: 165-175.
9. Yin, D., Z. Liu, D. Peng, Y. Yang, X. Gao, F. Xu and L. Han, 2013. Serum containing Tao-Hong-Si-Wu decoction induces human endothelial cell VEGF production via PI3K/Akt-eNOS signaling. Evidence-Based Complement. Altern. Med., Vol. 2013. 10.1155/2013/195158.
10. Wu, C.J., J.T. Chen, T.L. Yen, T. Jayakumar, D.S. Chou, G. Hsiao and J.R. Sheu, 2011. Neuroprotection by the traditional Chinese medicine, Tao-Hong-Si-Wu-Tang, against middle cerebral artery occlusion-induced cerebral ischemia in rats. Evidence-Based Complement. Altern. Med., Vol. 2011.10. 1155/2011/803015.
11. Han, L., Z. Ji, W. Chen, D. Yin and F. Xu *et al*., 2015. Protective effects of Tao-Hong-Si-Wu decoction on memory impairment and hippocampal damage in animal model of vascular dementia. Evidence-Based Complement. Altern. Med., Vol. 2015. 10.1155/2015/195835
12. Li, L., N. Yang, L. Nin, Z. Zhao and L. Chen *et al*., 2015. Chinese herbal medicine formula Tao Hong Si Wu decoction protects against cerebral ischemia-reperfusion injury via PI3K/Akt and the Nrf2 signaling pathway. J. Nat. Med., 69: 76-85.
13. Han, L., O. Qiao, H. Wu, S. Wu and Y. Zhang *et al*., 2017. Chromatographic fingerprint analysis is feasible for comprehensive quality control of Taohongsiwu. Int. J. Pharmacol., 13: 488-494.
14. Longa, E.Z., P.R. Weinstein, S. Carlson and R. Cummins, 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke, 20: 84-91.
15. Lu, T., Y. Jiang, Z. Zhou, X. Yue and N. Wei *et al*., 2011. Intranasal ginsenoside Rb1 targets the brain and ameliorates cerebral ischemia/reperfusion injury in rats. Biol. Pharmaceut. Bull., 34: 1319-1324.
16. Hatashita, S., J.T. Hoff and S.M. Salamat, 1988. Ischemic brain edema and the osmotic gradient between blood and brain. J. Cerebral Blood Flow Metab., 8: 552-559.
17. Zhao, Y., B. Fu, X. Zhang, T. Zhao, L. Chen, J. Zhang and X. Wang, 2014. Paeonol pretreatment attenuates cerebral ischemic injury via upregulating expression of pAkt, Nrf2, HO-1 and ameliorating BBB permeability in mice. Brain Res. Bull., 109: 61-67.
18. He, Y., H. Wan, Y. Du, X. Bie, T. Zhao, W. Fu and P. Xing, 2012. Protective effect of Danhong injection on cerebral ischemia-reperfusion injury in rats. J. Ethnopharmacol., 144: 387-394.
19. Durukan, A. and T. Tatlisumak, 2007. Acute ischemic stroke: Overview of major experimental rodent models, pathophysiology and therapy of focal cerebral ischemia. Pharmacol. Biochem. Behav., 87: 179-197.
20. Burtenshaw, D., M. Kitching, E.M. Redmond, I.L. Megson and P.A. Cahill, 2019. Reactive Oxygen Species (ROS), intimal thickening and subclinical atherosclerotic disease. Front. Cardiovasc. Med., Vol. 6. 10.3389/fcvm.2019.00089.
21. Naoi, M., Y. Wu, M. Shamoto-Nagai and W. Maruyama, 2019. Mitochondria in neuroprotection by phytochemicals: Bioactive polyphenols modulate mitochondrial apoptosis system, function and structure. Int. J. Mol. Sci., Vol. 20, No. 10. 10.3390/ijms20102451.

22. Maheshwari, A., L. Badgujar, B. Phukan, S.L. Bodhankar and P. Thakurdesai, 2011. Protective effect of Etoricoxib against middle cerebral artery occlusion induced transient focal cerebral ischemia in rats. Eur. J. Pharmacol., 667: 230-237.
23. Siesjo, B.K., 1992. Pathophysiology and treatment of focal cerebral ischemia: Part II: Mechanisms of damage and treatment. J. Neurosurg., 77: 337-354.
24. Liu, X., H. Chen, B. Zhan, B. Xing, J. Zhou, H. Zhu and Z. Chen, 2007. Attenuation of reperfusion injury by renal ischemic postconditioning: The role of NO. Biochem. Biophys. Res. Commun., 359: 628-634.
25. Dhalla, N.S., A.B. Elmoselhi, T. Hata and N. Makino, 2000. Status of myocardial antioxidants in ischemia-reperfusion injury. Cardiovasc. Res., 47: 446-456.
26. Powanda, D.D. and T.M.S. Chang, 2002. Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. Artif. Cells Blood Substitutes Biotechnol., 30: 23-37.
27. Gupta, S. and Y.K. Gupta, 2017. Combination of *Zizyphus jujuba* and silymarin showed better neuroprotective effect as compared to single agent in MCAo-induced focal cerebral ischemia in rats. J. Ethnopharmacol., 197: 118-127.
28. Chauhan, A., U. Sharma, K.H. Reeta, N.R. Jagannathan, R.D. Mehra and Y.K. Gupta, 2012. Neuroimaging, biochemical and cellular evidence of protection by mycophenolate mofetil on middle cerebral artery occlusion induced injury in rats. Eur. J. Pharmacol., 684: 71-78.
29. Gupta, Y.K., K. Sinha, G. Chaudhary and N.R. Jagannathan, 2002. Protective effect of adenosine against neuronal injury induced by middle cerebral artery occlusion in rats as evidenced by diffusion-weighted imaging. Pharmacol. Biochem. Behav., 72: 569-574.
30. Broughton, B.R.S., D.C. Reutens and C.G. Sobey, 2009. Apoptotic mechanisms after cerebral ischemia. Stroke, 40: e331-e339.
31. Dewson, G., 2015. Investigating Bax subcellular localization and membrane integration. Cold Spring Harbor Protoc., Vol. 2015, No. 5. 10.1101/pdb.prot086447.
32. Budihardjo, I., H. Oliver, M. Lutter, X. Luo and X. Wang, 1999. Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol., 15: 269-290.
33. Wang, X., 2001. The expanding role of mitochondria in apoptosis. Genes Dev., 15: 2922-2933.
34. Zhang, X., X. Xue, J. Zhao, C. Qian, Z. Guo, Y. Ito and W. Sun, 2016. Diosgenin attenuates the brain injury induced by transient focal cerebral ischemia-reperfusion in rats. Steroids, 113: 103-112.