Pharmacology, Biochemistry and Behavior 176 (2019) 33–42

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Tetramethylpyrazine-2′O-sodium ferulate provides neuroprotection against neuroinflammation and brain injury in MCAO/R rats by suppressing TLR-4/NF-κB signaling pathway

Peipei Zhou[a,1], Shuzhang Du[a,1], Lin Zhou[a,c], Zhi Sun[a,c], Li Hua Zhuo[a,c], Guangwei He[d], Yan Zhao[d], Yulin Wu[b,*], Xiaojian Zhang[a,c,**]

[a] Pharmaceutical department, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, 450052, China
[b] Department of Pharmacology, China Pharmaceutical University, Nanjing, PR China
[c] Laboratory of Accurate Clinical Pharmaceutical of Henan Province, Zhengzhou, Henan 450052, PR China
[d] Hefei Yigong Pharmaceutical Co., Ltd., Hefei, Anhui, PR China

ARTICLE INFO

Keywords:
MCAO
Ischemia
Neuroinflammation
Tetramethylpyrazine-2′O-sodium ferulate
TLR-4/NF-κB p65

ABSTRACT

Background: Neuroinflammation following cerebral ischemia is a serious risk factor in stroke patients. The purpose of this study was to investigate the neuroprotective effects of tetramethylpyrazine-2′O-sodium ferulate (TSF), a structurally modified compound from tetramethylpyrazine and ferulate, on cerebral ischemic injury and the underlying mechanisms.

Methods: Focal transient cerebral ischemia was induced in rat for 2 h by middle cerebral artery occlusion (MCAO) and the protective effect of TSF was studied using different doses of the drug (10.8, 18, 30 mg/kg, intravenously); Ozagrel (18 mg/kg) was used as the positive control. The drugs were given immediately after MCAO and the efficacy and mechanisms were evaluated at 72 h of reperfusion. The level of pro-inflammatory cytokines such as TNF-α, IL-1β and anti-inflammatory molecules such as IL-10 was measured; other factors such as neurological deficit, brain water content and infarct size and the level of MCP-1, ICAM-1, iNOS, CD11b, TLR-4/NF-κBp65 were also measured.

Results: TSF at the doses of 18, 30 mg/kg significantly improved neurological deficit, reduced brain water content and infarct size, accompanied by a decrease in the concentration of TNF-α, IL-1β, MCP-1, ICAM-1, iNOS and an increase in the concentration of IL-10. The amount of CD11b and ICAM-1 was found largely decreased and the expression of TLR-4 and the nuclear NF-κBp65 was weakened in TSF-treatment group.

Conclusions: Our study suggests that TSF possesses a neuroprotective effect against ischemic stroke which might be mediated through suppression of the inflammatory pathways in the brain following ischemic stroke.

1. Background

Ischemic stroke is the second most common cause of death and the most frequent cause of acquired disability in adult worldwide (Shukla et al., 2017). Decades of study on ischemic stroke has revealed a complex cascade of cytotoxic mechanisms, including oxidative stress, intracellular calcium-mediated excitotoxicity, inflammation and apoptosis, are activated following ischemic stroke (Lakhan et al., 2009; Moretti et al., 2015). Previous reports demonstrated that inflammation plays an important role in the pathogenesis of cerebral infarction (Huang et al., 2006). Toll-like receptors (TLRs), which play a central role in driving host inflammatory responses (Beutler, 2009; Iwasaki and Medzhitov, 2004), can be activated on microglia leading to the production of inflammatory mediators in the brain. TLRs dimerize and

Abbreviations: MCAO, middle cerebral artery occlusion; TSF, Tetramethylpyrazine-2′O-sodium ferulate; LCX, Ligusticum chuangxiong; TMP, tetramethylpyrazine; TLR-4, Toll-like receptor-4; NF-κB, nuclear factor-κB; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; iNOS, inducible nitric oxide synthase; Mac-1, macrophage-1 antigen

* Correspondence to: Y. Wu, Department of Pharmacology, China Pharmaceutical University, Nanjing 211198, PR China.
** Correspondence to: X. Zhang, Pharmaceutical Department, The First Affiliated Hospital of Zhengzhou University, Laboratory of Accurate Clinical Pharmaceutical of Henan Province, Zhengzhou, Henan, PR China.
E-mail addresses: wylcpu02@sina.com (Y. Wu), zhxjyxb18@126.com (X. Zhang).
[1] Peipei Zhou and ShuZhang Du contributed equally to this work.

https://doi.org/10.1016/j.pbb.2018.08.010
Received 22 March 2018; Received in revised form 17 August 2018; Accepted 28 August 2018
Available online 29 August 2018
0091-3057/

undergo conformational changes that lead to the activation of a complex cascade of intracellular signaling events, ultimately resulting in activation of the transcription factor nuclear factor-κB (NF-κB), which in turn regulates the expression of inflammatory responses (Kawai and Akira, 2007). The pathophysiologic evolution of stroke can be divided into three main phases. The first phase is characterized by energy failure that takes place at the center of an infarct within seconds to minutes. The second phase, in which the infarct grows, lasts up to 6-72 h after occlusion. During this period post ischemic microglial proliferation peaks at 48–72 h after focal cerebral ischemia. Which indicates the inflammation reaction reaches its most severe time, our experimental parameters were evaluated at 72 h reperfusion when the post-ischemic proliferation peaks. The third phase is the chronic phase that lasts for several days and weeks after injury, the phase of restoring and clearing the suffered tissue damage which is aimed at regaining lost functions to some extent.

Ligusticum chuangxiong (LCX) is a common component in traditional Chinese medicinal formulas and its decoctions have been used to treat several central nervous system diseases, suggesting its neuroprotective effects (Lin et al., 2009), tetramethylpyrazine (TMP) and Ferulic acid. Tetramethylpyrazine (TMP), a kind of amide alkaloids isolated from Ligusticum chuangxiong, whose biological functions involve vasodilatation and antiplatelet activity, is perhaps the most intensively studied compound for ischemic stroke, while Ferulic acid is known for its free radical scavenging property (Hintz and Ren, 2003). Tetramethylpyrazine-2′-O-sodium ferulate TSF is formed by the structure of tetramethylpyrazine and ferulic acid. Both of them are bioactive anti-inflammatory components of LCX. Many studies have shown that ligustrazine can dilate blood vessels, increase coronal and cerebral blood flow, inhibit platelet aggregation and decrease platelet activity and Ferulic acid is a non-peptide endothelin receptor antagonist, which can antagonize endothelin- induced vasoconstriction, reduce blood pressure. However, the half life of ligustrazine metabolism is fast and short, which is inconvenient for maintaining effective therapeutic concentration in clinical practice and the effect of ferulic acid is not ideal. Tetramethylpyrazine-2′-O-sodium ferulate TSF, a structurally modified compound improves the electrical properties by changing tetramethylpyrazine ring that contributes to increase the surface charges of platelets and erythrocytes obviously, which is favor of inhibiting of platelet aggregation, improving drug efficacy, enhancing the stability of the structure of medicine, thus slowing down the speed of drug metabolism and prolonging the time of action in vivo.

Previous studies have demonstrated that TSF could inhibit cerebral microcirculatory disturbance, neuron damage and cognitive impairment elicited by ischaemia (Xu et al., 2017) and our preliminary experiments results have come to a conclusion that TSF has a strong inhibitory effect on platelet aggregation via inhibiting thrombotic enzyme (TXA2) after cerebral ischemia-reperfusion, whereas there is no research on ischemic stroke through inhibition of neuro-inflammation of TSF. The aim of this study was to explore the anti-inflammatory and neuroprotective effects of TSF through its possible anti-inflammatory actions and to provide a new and novel drug with definite effect for ischemic stroke. In this experiment, ozagrel, a selective TXA2 synthase inhibitor (Ichikawa et al., 1999), was chosen as the positive drug since it is a commonly used drug in ischemia and its similar targets with TSF. In this study, we determined the modulatory effects of TSF on neurological deficit, cerebral tissue infarction, brain water content, infarct volume; CD11b, ICAM-1 expression were determined by immunohistochemistry; ELISA was employed for the estimation of TNF-α, IL-1β, IL-10, MCP-1, ICAM-1; fluorescence method was applied to INOS; Western blot (WB) was used for determination of TLR-4, NF-κB p65 in MCAO at 72 h reperfusion in rats after TSF treatment. We found that TSF regulated the inflammatory signaling in vivo by inhibiting the activation of NF-κB, and thus inhibiting the generation of the proinflammatory cytokines. To our knowledge, the relationship between TSF regulation of the inflammatory process and the changes in TLRs has not been defined. The study described herein was conducted to determine the effect of TSF on the TLRs/NF-κB p65 signaling pathway of ischemic reperfusion in rats.

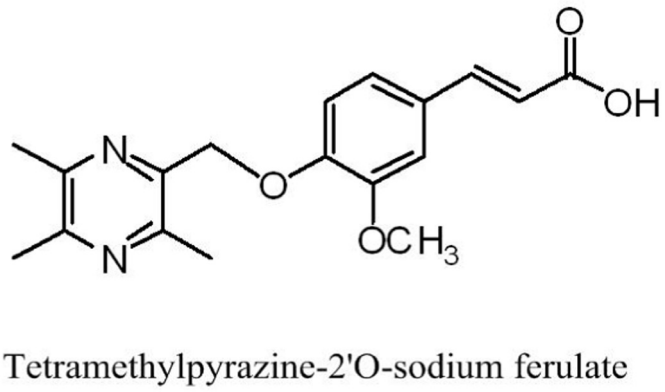


Fig. 1. Chemical structure of TSF (Tetramethylpyrazine-2′O-sodium ferulate).

2. Methods

2.1. Animals and drugs

Adult male Sprague-Dawley rats (250–280 g) were purchased from Qinglongshan Animal Center (Nanjing, China). Rats were maintained under a 12 h/12 h light/dark cycle with free access to food and water. TSF was provided by Hefei Medical Pharmaceutical Co. Ltd (Hefei, China). All procedures used in this experiments were carried out in adherence with the guidelines of the Institutional Animal Care and Use Committee of China and followed the guidelines of the regional Animal Ethics Committee of China Pharmaceutical University (Fig. 1).

2.2. Cerebral ischemia/reperfusion model

The standard MCAO rat model was established according to the methods of originally described by Longa et al. (Longa et al., 1989). and our previous study. Briefly, Male SD rats were anesthetized with 3% chloral hydrate (1 ml/100 g) intraperitoneally, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated and exposed. Then a nylon monofilament approximately 0.24 mm in diameter with a rounded tip was inserted through ECA into the ICA to block the origin of MCA until the slightly resistance was felt. Cerebral ischemia was maintained for 2 h, then put off the suture slowly to achieve 72 h reperfusion, meanwhile, body temperature of rats were maintained at 36.5–37.5 °C with a thermostat-controlled heating pad. The sham operated group rats were underwent the same surgical procedures without inserting a filament.

2.3. Drug administration and groups

TSF was dissolved in physiological saline to prepare concentrations of 10.8, 18 and 30 mg/ml and rats were administered intravenously once a day after operation, 60 rats were used to evaluate of neurological deficits, cerebral infarct volume and brain water content, 60 rats were employed for the detection of inflammatory cytokines and adhesion molecules, six rats for Immunohistochemical in each group and four for Western blot. Following 7 days acclimatization, rats were randomly segregated into six groups: sham control group, for which the incision was made but filament was not inserted and this group received a dose of 0.9% saline; the ischemic reperfusion (IR) group, which received MCAO and also received 0.9% saline; TSF groups, which received MCAO and received 10.8 mg/kg (low dose), 18 mg/kg (medium dose), 30 mg/kg (high dose) of TSF; and positive control group, which received MCAO and 18 mg/kg (Ozagrel). TSF was dissolved in 0.9%

saline, and the intravenous injection volume of TSF and Ozagrel was 0.2 ml/100 g of body weight.

2.4. Evaluation of neurological deficit

The groups were examined for neurological deficit in a blinded manner at 72 h post-ischemia and it was scored using a four-point scale (0: no neurological deficit with normal spontaneous movements, 1: the left front was flexed and failure to extend the left forepaw completely, 2: circling to the left constantly, 3: falling to the left and crawling slowly, 4: no spontaneous walking with no response to noxious stimulus). After this evaluation, animals were sacrificed immediately by decapitation and submitted to the next tests.

2.5. Assessment of cerebral infarct volume

The brain was removed and kept at −20 °C for 40 min. Then the frozen brains were sliced into uniform coronal sections of approximately 2 mm thickness each using 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, Austria) in 0.9% saline at 37 °C incubation for 15 min, then slices were photographed. The unstained (white) areas indicated infracted tissue and the stained (red) areas indicated normal tissue. The infracted tissue areas were analyzed by weighing the infarct area, and infarct area content was calculated as infarct weight / (infarct area + normal area weight) × 100%.

2.6. Determination of brain water content

For brain water content measurement, brain coronal sections were cut at a thickness of 2 mm and each slice was divided into ipsilateral and contralateral hemispheres. The two hemispheres slices, packaged with tin foils, were weighed and dried for 24 h at 100 °C to calculate the dry weight. Brain water content was calculated as (wet weight − dry weight) / wet weight × 100%.

2.7. Measurements of TNF-α, IL-1β, and IL-10 concentrations by ELISA

To determine TNF-α, IL-1β, and IL-10 concentrations in the ischemic brain, the cortex was homogenized in 0.9% cold saline and then centrifuged at 3000 ×*g* for 15 min at 4 °C. The supernatant was collected and 10% homogenate was used to determine TNF-α, IL-1β and IL-10 concentrations at 460 nm as described by the assay kit (Suzhou Calvin Biotechnology Co., Ltd., Nanjing, China). The level of the molecules was expressed as pg/mg of wet tissue.

2.8. Detection of MCP-1, ICAM-1 and iNOS

Tissue samples were disposed as described above and the level of MCP-1, ICAM-1 was determined. The measurements were performed as described by the assay kits (Suzhou Calvin Biotechnology Co., Ltd., Nanjing, China). Nitric oxide synthase activity(iNOS) based on fluorescence method was determinated by multi-mode Microplate Reader using Nitric Oxide Synthase Assay Kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). The content was expressed as pg/mg protein for MCP-1, and ICAM-1 and U/mg protein for iNOS.

2.9. Immunohistochemical determination of CD11b, and ICAM-1

Paraffin-embedded sections were used to assess the expression of CD11b, and ICAM-1 according to standard histological procedures. We chose Rabbit Anti-CD11b antibody (bs-1014R) and Rabbit Anti-ICAM1/CD54 antibody (bs-0608R) (Beijing Biosynthesis Biotechnology Co., Ltd.) to measure the expression of the proteins. The immunoreactive cells were detected under a 200 × light microscope in three visual fields of the ischemic cortex region of the infarct.

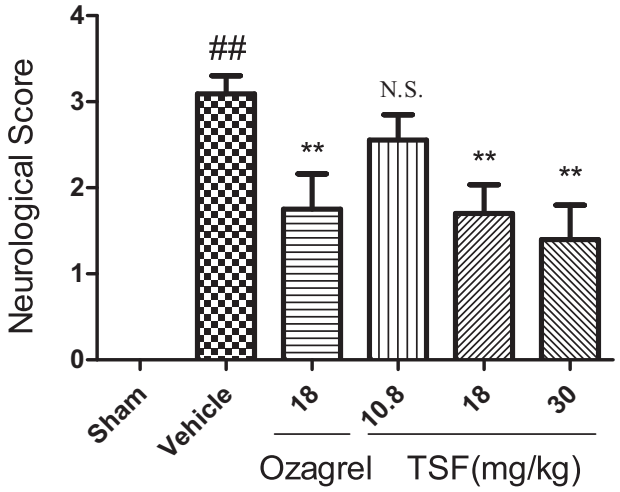


Fig. 2. Effects of TSF on neurological deficit scores at 72 h reperfusion after MCAO. The evaluation of neurological deficits was measured by four-point scale (0–4). Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group, N.S. stands for "no significance".

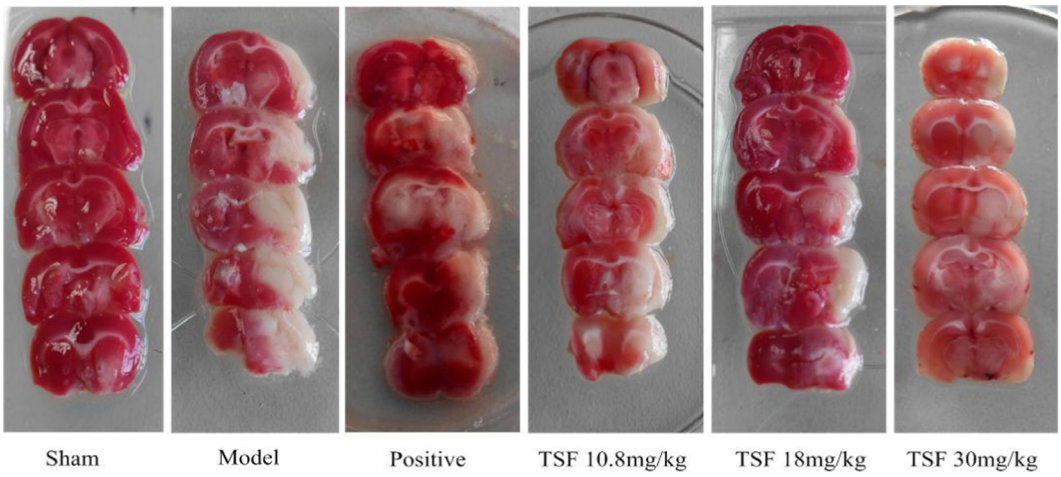
2.10. Western blot of TLR-4/NF-κB p65

To examine the inhibitory effects of TSF on TLR-4/NF-κB p65 pathway following MCAO, the protein expressions of TLR-4, and the nuclear NF-κBp65 were analyzed. The tissues from the cerebral cortex and hippocampus of brain were obtained at 72 h after MCAO (n = 4 for each group) and homogenized in RIPA buffer containing PMSF, the nuclear proteins NF-κB p65 was prepared by using nuclear protein extraction kit (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, the brain tissues were homogenized in ice-cold hypotonic buffer followed by centrifugation at 4 °C, 3000*g* for 5 min. After addition of appropriate volume of lysis buffer, the lysate was homogenized and centrifuged at 4 °C, 15,000 g for 10 min to obtain supernatant nucleoprotein which was employed to detect the level of nuclear NF-κB p65 with Histone H3 as an inner control. The isolated protein supernatant was measured using a BCA Protein Assay reagent kit (Beyotime Biotechnology, Shanghai China). Equal quantities of protein samples was separated by 6% to 12% SDS/PAGE and transferred on to PVDF membranes. Membranes were blocked with BSA and probed with primary antibodies TLR-4 (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κB (Cell Signaling Technology, Boston, MA, USA) at 4 °C overnight. The membranes were washed three times with Tris-buffer saline containing 0.05% Tween 20 (TBST) buffer and were probed with secondary antibodies (anti-mouse IgG) coupled to horseradish peroxidase (HPR) (1:5000; Abgent Biotechnology, CA, USA) for 2 h at room temperature. Protein bands were visualized with enhanced chemiluminescence kit (Thermo, IL, USA). The protein bands were quantified using the electrochemiluminescence detection system (Tannon-5200, Shanghai, China) as per the instructions of conventional protocol and the relative protein expressions were expressed as the ratios of TLR-4, and NF-KB p65; β-actin, Histone-H_3 was used as the loading control for TLR-4 and nuclear NF-KB p65 respectively.

2.11. Statistical analyses

Quantitative data were expressed as mean ± standard deviation (S.D.) using statistical software SPSS19.0. Differences were evaluated by one-way analysis of variance (ANOVA) followed by a least significant difference *t*-test (LSD). Differences were considered significant at P < 0.05.

A)



B)

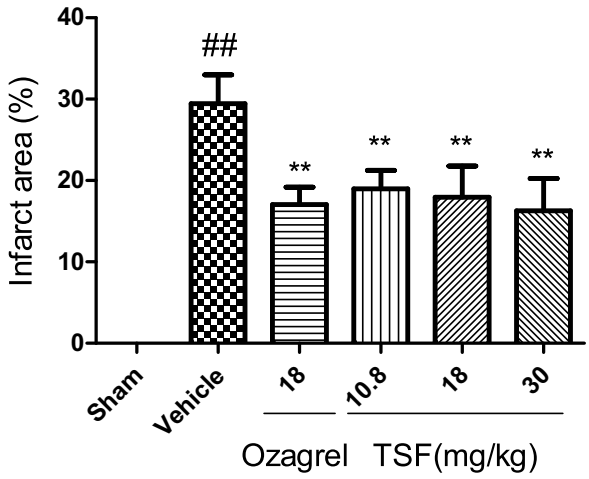


Fig. 3. Effects of TSF on brain infarction at 72 h reperfusion after MCAO. A) Representative images of TTC staining in different groups of TSF at 72 h after I/R. Red areas represent normal tissue, while the white is infarction. B) Quantitative analysis of infarct size in different groups of TSF. Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. TSF improved neurological deficit

Animals in the IR group increased significantly neurological deficit MCAO: (3.09 ± 0.71%) than Sham-operated group (P < 0.01). The medium 18 mg/kg (1.70 ± 1.05%) and high doses 30 mg/kg (1.40 ± 1.26%) of the TSF ameliorated neurological deficits during ischemia, versus the model group (P < 0.01, P < 0.01); however, no significant difference was seen in the low dose group 10.8 mg/kg (2.55 ± 0.88%) compared to Model group (Fig. 2).

3.2. TSF reduced infarct volume

About 30.0% of severe infarct volume was observed in the MCAO/R group when compared to the sham-operated group (P < 0.01). Among the other groups, treatments with TSF at all doses significantly reduced the infarct volume and showed dose-dependent reductions compared with the control group. Infarct volumes after treatment with 10.8, 18 and 30 mg/kg TSF were reduced approximately by 35.6% (18.97 ± 2.28%), 39.1% (17.94 ± 3.83%), and 44.8% (16.27 ± 3.99%), respectively, versus the model group (29.45 ± 3.54%). Compared to the positive group of 17.05 ± 2.13%, the protective effects of TSF at 18 mg/kg reached the same effect as that of the positive group, and the high dose showed better efficacy than the positive control Ozagrel (Fig. 3).

3.3. TSF reduced brain edema

TSF significantly decreased water content compared to the animals in model group, especially with the administration of medium and high TSF dose (medium 79.93% ± 2.09%; high 78.00% ± 2.82%; MCAO: 84.08% ± 2.58%, P < 0.05). However, low dose had no evident difference on water content. TSF medium dose showed equal effectiveness to positive control drug (positive vs. medium: 79.75% ± 1.52% vs. 79.93% ± 2.09%) (Fig. 4).

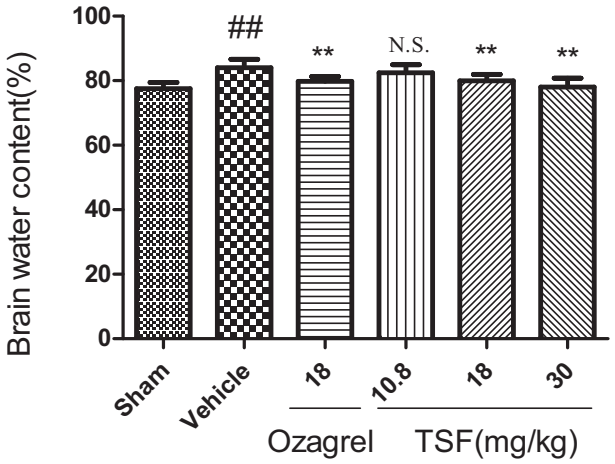


Fig. 4. Effects of TSF on brain water content at 72 h reperfusion after MCAO. Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group, N.S. stands for "no significance".

3.4. Effects of TSF on the levels of TNF-α, IL-1β, and IL-10 in the injured brain after MCAO

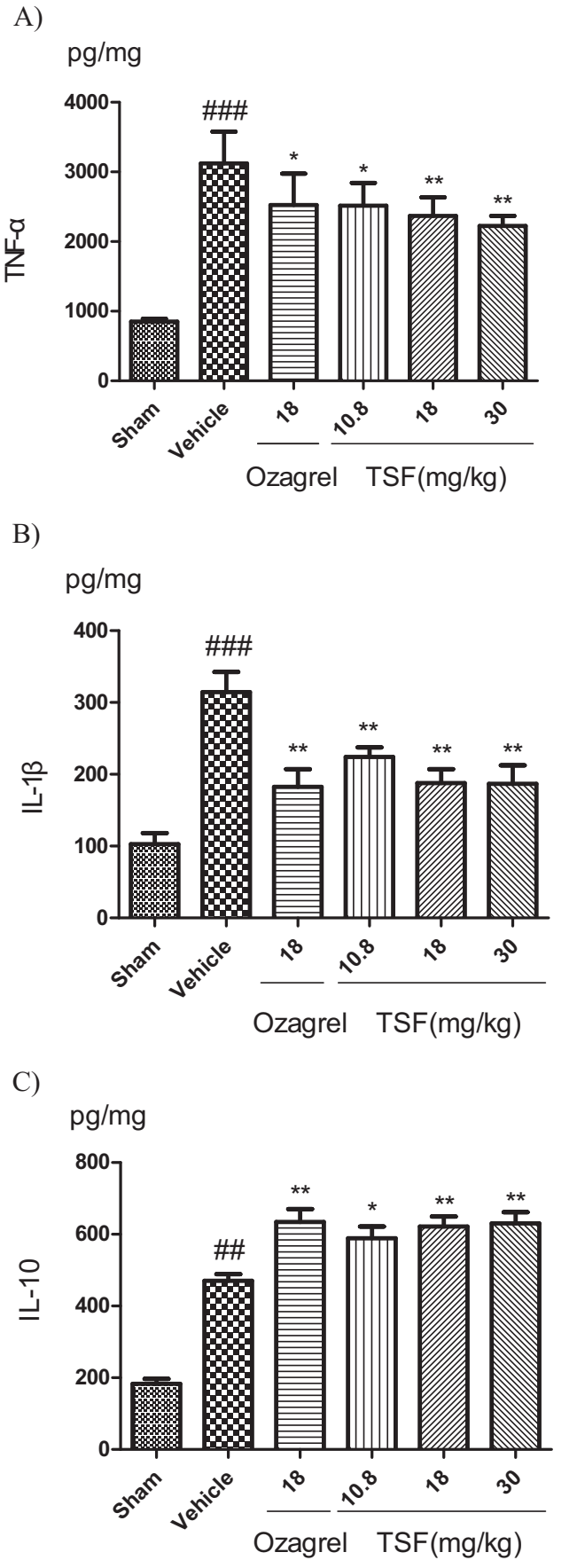
ELISA assay showed that the MCAO group had significantly higher expressions of pro-inflammatory mediators, such as TNF-α, and IL-1β than did the sham group (P < 0.05). In comparison with the model group, TSF treatment (18 mg/kg/day, 30 mg/kg/day) significantly decreased TNF-α, and IL-1β concentration in the cerebral cortex and hippocampus of brain tissue (P < 0.05); compared to the positive control group, the efficacy of the medium dose was better in this aspect. On the other hand, expression of the anti-inflammatory mediator IL-10 was higher in the MCAO group than the sham group. Expression of IL-10 was observed to be significantly up-regulated in TSF groups. Treatment with TSF, especially the medium and high doses, significantly up-regulated IL-10 level (Fig. 5).

3.5. Effects of TSF on the levels of MCP-1, ICAM-1, and iNOS in the injured brain after MCAO

MCAO/R rats showed a significant increase in MCP-1 level than sham-operated group. And MCP-1 level was found to be decreased significantly in the 18 and 30 mg/kg-treated TSF groups than the model rats (P < 0.01); The level of ICAM-1 was significantly increased in MCAO/R group, and suppressed by treatment of TSF medium and high dose (P < 0.01); the positive control and the low dose also attenuated the expression of ICAM-1 but the effect was not as strong as the medium and high dose, but the data still have statistical differences (P < 0.05). Nitric oxide synthase was also increased in the MCAO/R rats (P < 0.01, compared to the Sham-operated group). Among the drug treatment groups, the medium and high administration of TSF greatly reduced the nitric oxide synthase content than the MCAO/R group, but 10.8 mg/kg TSF did not have significant effect (Fig. 6).

3.6. TSF reduced positive Immunoreactive cells of CD11b and ICAM-1

To determine the expression of CD11b and ICAM-1 in the rat ischemic cerebral cortex and hippocampus before and after treatment, we performed immunohistochemical analyses of brain sections. CD11b antigen was expressed in recruited leukocytes and resident microglia (Cheng et al., 2008), ICAM-1 immunoactivity was presented on endothelium. The amount of CD11b and ICAM-1 were found little in the sham group, however these significantly increased in MCAO at 72 h of reperfusion. (P < 0.05 vs. Sham group). Compared with MCAO group,



(caption on next page)

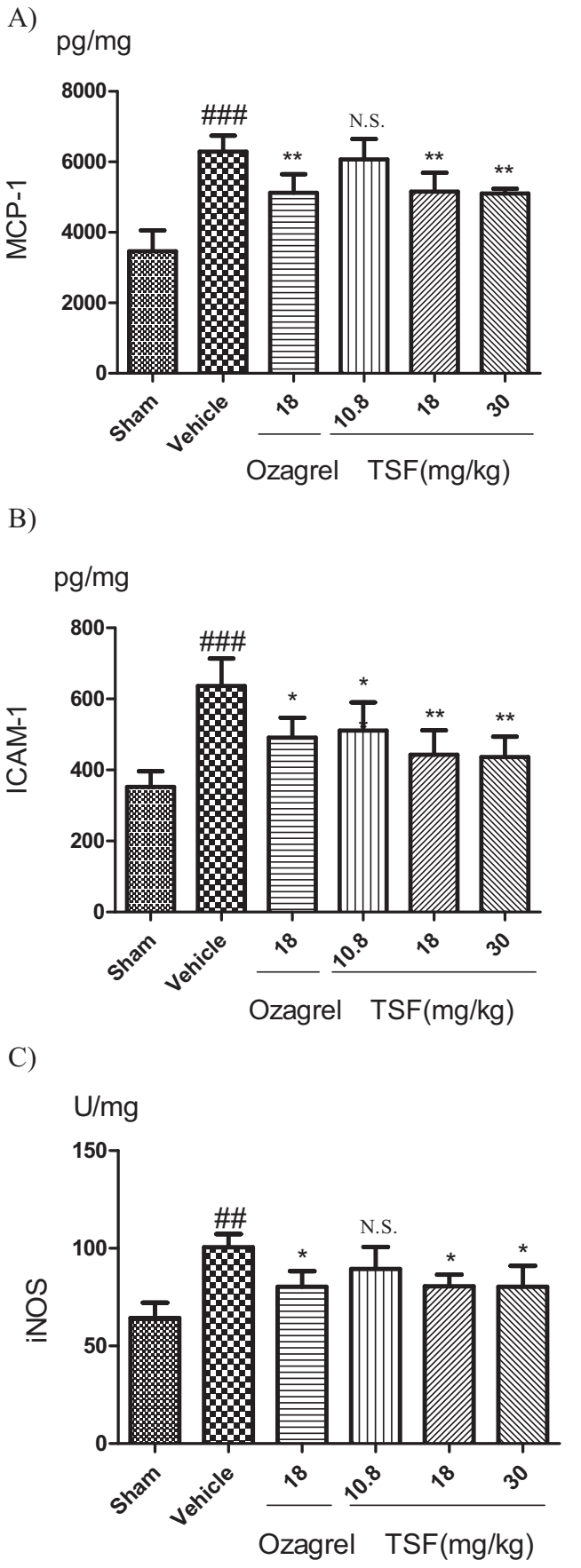


Fig. 5. Results of TSF treatment in the cerebral cortex at 72 h after MCAO-induced inflammation A) TNF-α, B) IL-β, C) IL-10. Inflammatory cytokines were detected by ELISA kits following manufacturer's procedure. Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group.

TSF decreased the positive cells of CD11b and ICAM-1 (Figs. 7–8) in the ischemic cortex (P < 0.05), especially the medium and the high TSF groups dramatically lowered the expression the CD11b and ICAM-1 with comparison of MCAO group.

3.7. TSF decreased the expression of TLR-4 and NF-κB p65

To investigate the further mechanism, we used WB to examine the expression of nuclear NF-κB p65 and TLR-4 (Figs. 9–10). WB analysis revealed a significant increase in NF-κB p65 and TLR-4 nuclear in the MCAO/R rats compared to the sham group and the results indicated that the expressions of both nuclear NF-κB p65 and TLR-4 were markedly up-regulated by 114.47% and 147.02% respectively. After intravenous administration of TSF, especially 18 mg/kg and 30 mg/kg TSF per day at 72 h of reperfusion notably inhibited the expression of the expression of nuclear NF-κB p65 and TLR-4 compared with the MCAO group (P < 0.01, P < 0.01), meanwhile the results showed there are also greatly differences between the low dose TSF and model group on nuclear NF-κB p65, as well as the expression of TLR-4.

4. Discussion

It has been reported that inflammation plays an important role in the pathogenesis of central nervous system injury and there is increasing evidence that post-ischemic inflammation contributes to ischemic brain injury (Feuerstein et al., 1998; Deb et al., 2010). In this study we found that TSF improved the functional recovery following MCAO, as proved by decreased neurological deficit score and infarct volume. Furthermore, TSF increased anti-inflammatory mediator IL-10 and decreased pro-inflammatory mediators TNF-α, and IL-1β in the cerebral cortex, which was likely associated with restorative effects of TSF; TSF also reduced the expression of MCP-1, ICAM-1, and INOS. TSF attenuated these inflammatory cascades by possibly suppressing the TLR-4/NF-κB p65 pathways as observed in our WB experiments. Another mechanism that controls TLR-induced neuro-inflammation and injury may involve negative feedback inhibitors of TLRs and inflammatory cytokines. It was hypothesized that in cerebral ischemia, endogenous signals are generated that trigger a mild inflammatory response and induce the production of inflammatory inhibitors. This process would limit the inflammatory response to a subsequent event of severe ischemia (Kariko et al., 2004; Lambertsen et al., 2012). IL-10 is one of the suggested negative regulators, which blocks the production of inflammatory cytokines (Moore et al., 2001; Park et al., 2012) and downregulates TLR-4 expression. In the current study, negative regulation of TLR-4/NF-κB p65 through the increasing expression of IL-10 could be one of the ameliorative mechanisms by which TSF mediated its effect in the injured brain.

The Pathobiology of ischaemic stroke develops from a complex series of pathophysiological events that involves the complex interplay of multiple pathways including: excitotoxicity, acidotoxicity and ionic imbalance, peri-infarct depolarization, oxidative and nitrative stress, inflammation and apoptosis (Fann et al., 2013). Inflammation plays a crucial role in the overall pathogenesis of ischemic stroke, it is now widely known that the inflammatory reaction following by cerebral ischaemia result in the progression of brain injury and exacerbation of focal neurological deficits. Inflammation is an innate immune response to infection and tissue damage triggered by the detection of acute damage through extracellular and intracellular pattern recognition receptors (PRRs). The toll-like receptors (TLRs) are important receptors of

(caption on next page)

Fig. 6. Results of TSF treatment in the cerebral cortex at 72 h after MCAO-induced inflammation A) MCP-1, B) ICAM-1, C) iNOS. The inflammatory adhesion, chemokine were detected by ELISA kits following manufacturer's procedure; Nitric oxide synthase were measured by fluorescence method. Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group, N.S. stands for "no significance".

the innate immune system, acting as indicators of tissue injury and mediating inflammatory responses to brain damage that expressed on endothelial cells, microglia, astrocytes and neurons Within the CNS function, TLRs as a first-line defense against pathogen invasion which recognize pathogen-associated molecules such as lipopolysaccharide (TLR4). Activation of TLRs contributes to an activation of proinflammatory transcription factors NF-kB, one of the primary downstream signals that upregulate cytokine and chemokine production initiating a localized inflammatory response (Takeuchi and Akira, 2010). The inflammatory response is characterized by the production and release of pro-inflammatory cytokines which are important molecular signals released in the injured brain quickly after ischaemic insult. Among those tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) activated by cells including neurons, astrocytes, microglia and endothelial cells contribute greatly to neuronal cells and glial cell death during cerebral ischemia (Rakoff-Nahoum and Medzhitov, 2008). Consequently, the expression of adhesion molecules on the surface of endothelial cells, leukocytes, and platelets elicited by pro-inflammatory cytokines such as intercellular adhesion molecule-1(ICAM-1), selectins, and integrins Mac-1. These adhesions are crucial for the interaction between the endothelium and blood-borne leukocytes, adhesion molecules interact with complementary surface receptors on leukocytes which in turn adhere to the endothelium of postcapillary venules, followed by transmigration of leukocytes into the brain parenchyma, what is the process of leukocyte infiltration into brain tissue (Kunz et al., 2010). In addition, monocyte chemoattractant protein-1 (MCP-1) released by activated neurons and glial cells, the major chemokine in mammalian systems, is important in guiding leukocyte migration towards the damaged tissue (Peruzzotti-Jametti et al., 2014). Meanwhile, coinciding with leukocyte infiltration, a variety of pro-inflammatory cytokines along with toxic metabolites are emerging by activated microglia, the production of toxic mediators play a vital role to brain damage, it has been demonstrated that nitric oxide synthase (iNOS), an enzyme that that generates toxic amounts of nitric oxide (NO), and release both matrix metalloproteinases (MMPs), NO is a significant molecule that would worsen infarct volumes as well as neurologic damage. Release of MMP-9 contribute to the further down-regulation of junctional proteins and are the main contributors to the first derangement of the BBB (McColl et al., 2009).

In general, a robust inflammatory reaction following focal cerebral ischemia releases inflammatory signaling molecules, adhesion molecules and transcriptional regulators as key elements (Danton and Dietrich, 2003; Wang et al., 2007). Several cell types contribute to post-ischemic inflammation. First of all, the activated microglia and astrocytes secrete inflammatory factors such as cytokines, chemokines and inducible nitric oxide synthase (Che et al., 2001; Hewett et al., 1996).

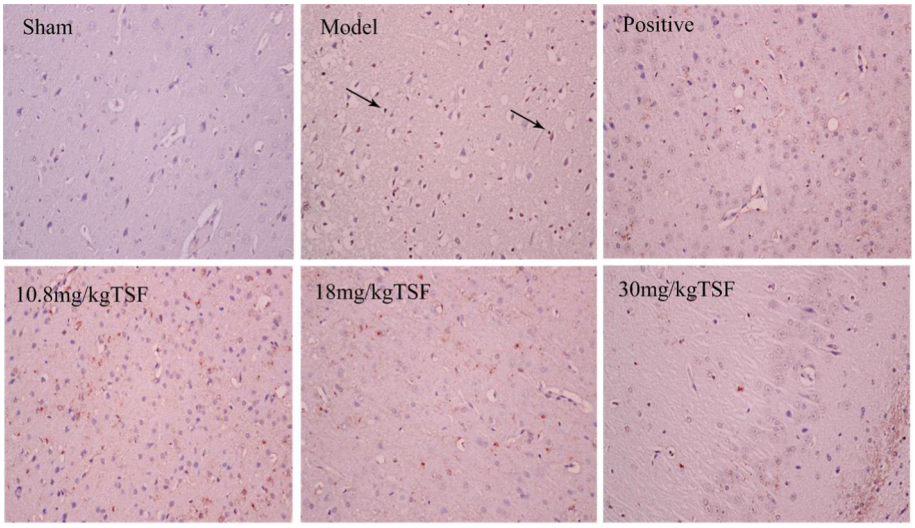
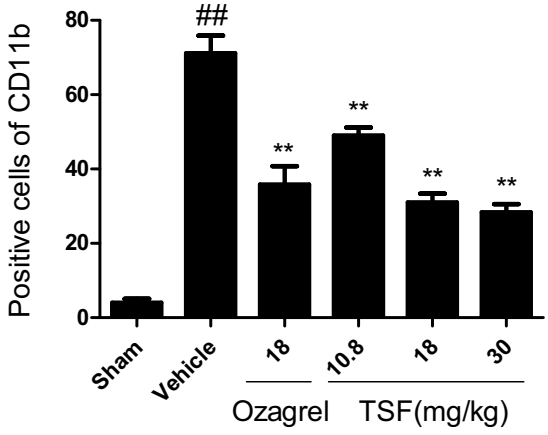


Fig. 7. Representative photographs depicted the expression of CD11b in the cerebral cortex and hippocampus at 72 h reperfusion after MCAO. CD11b immunoactivity was presented in recruited leukocytes and microglia (black arrows indicate positive CD11b cells, the positive products were brown granules after immunostaining), which was largely increased after MCAO and was significantly decreased after treatment with TSF. Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



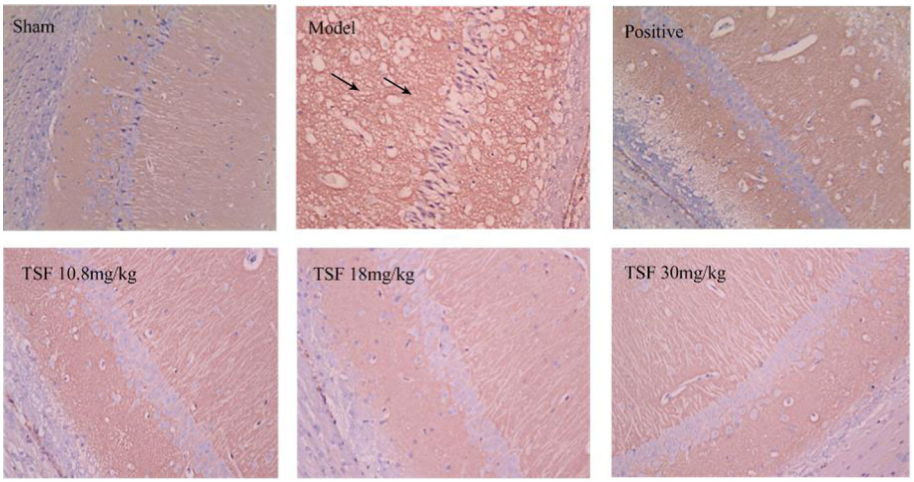
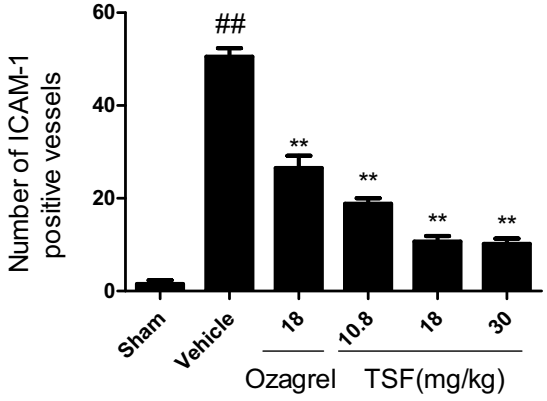


Fig. 8. Representative photographs depicted the expression of ICAM-1 in the cerebral cortex and hippocampus at 72 h reperfusion after MCAO. ICAM-1 immunoactivity was presented on endothelium. The appearance of brown or brown granular deposition in the cytoplasm and the intercellular of ICAM-1 immunostaining were performed in order to visualize the interaction and between neutrophils and endothelium. Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



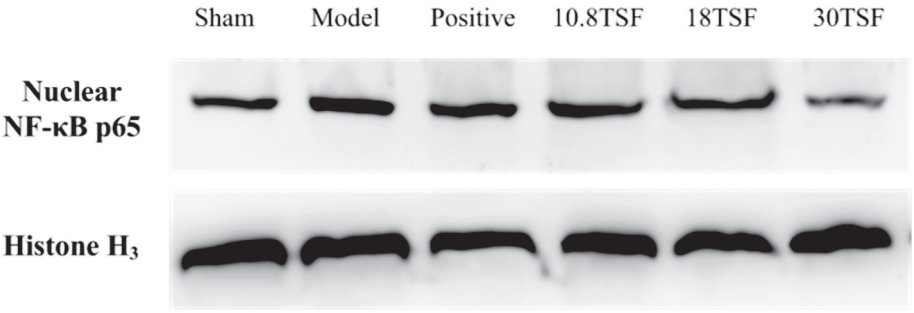
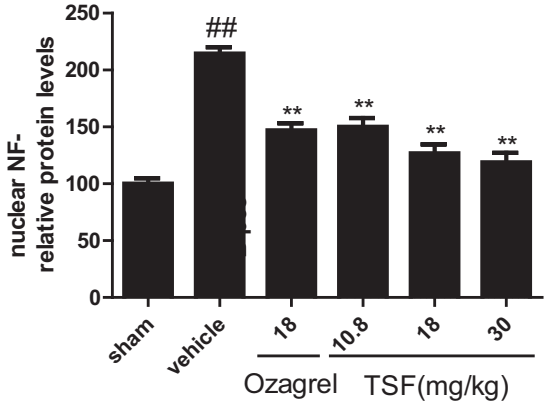


Fig. 9. Western blot analysis of nuclear NF-κB p65 in the cerebral cortex and hippocampus of rats at 72 h after MCAO. Representative images of nuclear NF-κB p65 protein are present. The protein levels were expressed as a ratio of the Histone H_3 levels. Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group.



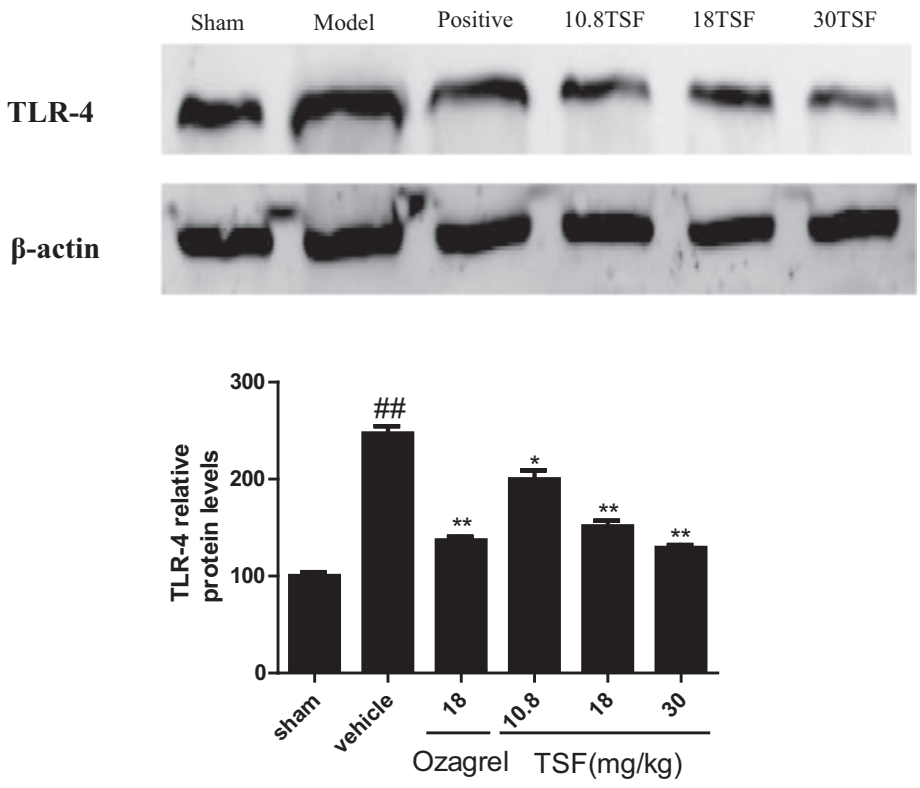


Fig. 10. Western blot analysis of TLR-4 in the cerebral cortex and hippocampus of rats at 72 h after MCAO. Representative images of TLR-4 protein are present. The protein levels were expressed as a ratio of the β-actin levels. Data were expressed with mean values ± standard deviation (SD). ##P < 0.01 versus Sham group, **P < 0.01 *P < 0.05 versus TSF group.

Upon activation following ischemia, within 4–6 h after ischemic onset, circulating leukocytes adhere to vessel walls and migrate into the brain with subsequent release of more pro-inflammatory mediators (Hallenbeck, 1996). Cytokines are important inflammatory mediators that are produced by immune cells and resident brain cells after ischemia. Increased production of pro-inflammatory cytokines and lower levels of the anti-inflammatory IL-10 are related to larger infarctions (Vila et al., 2003). Cytokines elicit the synthesis of ICAM-1 and adhesion molecules play a pivotal role the infiltration of leukocytes into the brain parenchyma (Degraba, 1998; Khan et al., 2007). Also chemokines are important for cellular communication and inflammatory cell recruitment. Expression of chemokines such as MCP-1 is thought to have a deleterious effect by increasing leukocyte infiltration (Stamatovic et al., 2003; Guo et al., 2012). Meanwhile microvascular obstruction by neutrophils can worsen the degree of ischemia, production of toxic mediators by activated inflammatory cells and injured neurons also has important consequences. In rodent models of cerebral ischemia, infiltrating neutrophils produce inducible NOS (iNOS), an enzyme that produces toxic amounts of NO (Forster et al., 1999) as seen in patients with stroke (Askalan et al., 2006).

Our previous studies have demonstrated that TSF significantly reduced BBB permeability and brain oedema which were correlated with regulating the expression of TJ proteins, MMP-9 and AQP4 (Xu et al., 2017). The purpose of the present study was to examine whether treatments with TSF have anti-inflammation neuroprotection in rats undergoing transient focal cerebral ischemia followed by 72 h reperfusion. According to our observations, 18 and 30 mg/kg TSF significantly decreased neurological deficit scores, infarct volume, brain water content and the inflammatory mediators. And the levels of adhesion molecule ICAM-1, chemokine MCP-1, and toxic mediator iNOS were notably depressed after the treatment of TSF, the dose of 18 mg/kg and 30 mg/kg doses were shown effectiveness in our experimental animals through its neuroprotective roles in ischemic stroke, while the 10.8 mg/kg dose failed to exhibit the significant effect presented in some particular data from results above.

CD11b, a subunit of macrophage-1 antigen (Mac-1, CD11b/CD18) which is a heterodimeric protein, is expressed on the surface of activated leukocytes (Arumugam et al., 2004; Hickstein et al., 1993). CD11b also acts as an activation maker for microglia (Nakase et al., 2004), which release a range of neurotoxic compounds including nitric oxide (NO), cytokines, and chemokines (Kao et al., 2006; Allen and Bayraktutan, 2009). In our results, immunohistochemistry was performed to examine the immunoreactivity of CD11b and ICAM-1, the positive cells of CD11b and ICAM-1 observed were significantly decreased with TSF treatment, with the medium and high doses having obvious effect. This indicated TSF may have attenuated the activation of leukocytes and microglia to alleviate the injured brain tissue (Arumugam et al., 2004; Nakase et al., 2004).

To further explore the protective mechanisms of TSF, we studied NF-κB signal transduction pathways known to be activated by TLR-4. TLR-4 signaling plays a significant and detrimental role in brain ischemia (Kacimi et al., 2011; Buchanan et al., 2010). TLR signaling pathways culminate the activation of the transcription factor NF-κB (Xiang et al., 2018), which controls the expression of an array of inflammatory cytokine genes (Beutler, 2009; Baker et al., 2011). From our results of WB of TLR-4 and NF-κB p65, TSF seemed to weaken the expression of TLR-4 and the NF-κB p65 greatly.

5. Conclusions

In conclusion, our study demonstrated that treatment with TSF could alleviate the injured brain following MCAO via improvement of neurological deficit, reduction of brain edema and by decreasing the levels of chemokines, adhesion molecules, inducible nitric oxide synthase and inflammatory cytokines and increasing anti-inflammatory molecules, through the suppression of TLR/NF-κB p65 signaling pathway. Our results suggest TSF as a new therapeutic agent for the treatment of ischemic stroke.

Acknowledgments

We acknowledge the grant and drug support from Hefei Yigong

Pharmaceutical Institute Co., Ltd., Hefei, PR China and China Pharmaceutical University, Nanjing, PR China.

Conflicts of interest

The authors declare that there is no conflict of interest.

References

Allen, C.L., Bayraktutan, U., 2009. Oxidative stress and its role in the pathogenesis of ischaemic stroke. Int. J. Stroke 4 (6), 461–470.

Arumugam, T.V., Salter, J.W., Chidlow, J.H., Ballantyne, C.M., Kevil, C.G., Granger, D.N., 2004. Contributions of LFA-1 and Mac-1 to brain injury and microvascular dysfunction induced by transient middle cerebral artery occlusion. Am. J. Physiol. Heart Circ. Physiol. 287 (6), H2555–H2560.

Askalan, R., Deveber, G., Ho, M., Ma, J., Hawkins, C., 2006. Astrocytic-inducible nitric oxide synthase in the ischemic developing human brain. Pediatr. Res. 60 (6), 687–692.

Baker, R.G., Hayden, M.S., Ghosh, S., 2011. NF-kappaB, inflammation, and metabolic disease. Cell Metab. 13 (1), 11–22.

Beutler, B.A., 2009. TLRs and innate immunity. Blood 113 (7), 1399–1407.

Buchanan, M.M., Hutchinson, M., Watkins, L.R., Yin, H., 2010. Toll-like receptor 4 in CNS pathologies. J. Neurochem. 114 (1), 13–27.

Che, X., Ye, W., Panga, L., Wu, D.C., Yang, G.Y., 2001. Monocyte chemoattractant protein-1 expressed in neurons and astrocytes during focal ischemia in mice. Brain Res. 902 (2), 171–177.

Cheng, C.Y., Su, S.Y., Tang, N.Y., Ho, T.Y., Chiang, S.Y., Hsieh, C.L., 2008. Ferulic acid provides neuroprotection against oxidative stress-related apoptosis after cerebral ischemia/reperfusion injury by inhibiting ICAM-1 mRNA expression in rats. Brain Res. 1209, 136–150.

Danton, G.H., Dietrich, W.D., 2003. Inflammatory mechanisms after ischemia and stroke. J. Neuropathol. Exp. Neurol. 62 (2), 127–136.

Deb, P., Sharma, S., Hassan, K.M., 2010. Pathophysiologic mechanisms of acute ischemic stroke: an overview with emphasis on therapeutic significance beyond thrombolysis. Pathophysiology 17 (3), 197–218.

Degraba, T.J., 1998. The role of inflammation after acute stroke: utility of pursuing anti-adhesion molecule therapy. Neurology 51 (Suppl. 3), S62–S68.

Fann, D.Y., Lee, S.Y., Manzanero, S., Chunduri, P., Sobey, C.G., Arumugam, T.V., 2013. Pathogenesis of acute stroke and the role of inflammasomes. Ageing Res. Rev. 12 (4), 941–966.

Feuerstein, G., Wang, X., Barone, F.C., 1998. Cytokines in brain ischemia—the role of TNF alpha. Cell. Mol. Neurobiol. 18 (6), 695–701.

Forster, C., Clark, H.B., Ross, M.E., Iadecola, C., 1999. Inducible nitric oxide synthase expression in human cerebral infarcts. Acta Neuropathol. 97 (3), 215–220.

Guo, R.B., Wang, G.F., Zhao, A.P., Gu, J., Sun, X.L., Hu, G., 2012. Paeoniflorin protects against ischemia-induced brain damages in rats via inhibiting MAPKs/NF-kappaB-mediated inflammatory responses. PLoS One 7 (11), e49701.

Hallenbeck, J.M., 1996. Significance of the inflammatory response in brain ischemia. Acta Neurochir. Suppl. 66, 27–31.

Hewett, S.J., Misko, T.P., Keeling, R.M., Behrens, M.M., Choi, D.W., Cross, A.H., 1996. Murine encephalitogenic lymphoid cells induce nitric oxide synthase in primary astrocytes. J. Neuroimmunol. 64 (2), 201–208.

Hickstein, D.D., Grunvald, E., Shumaker, G., Baker, D.M., Back, A.L., Embree, L.J., Yee, E., Gollahon, K.A., 1993. Transfected leukocyte integrin CD11b/CD18 (Mac-1) mediates phorbol ester-activated, homotypic cell:cell adherence in the K562 cell line. Blood 82 (8), 2537–2545.

Hintz, K.K., Ren, J., 2003. Tetramethylpyrazine elicits disparate responses in cardiac contraction and intracellular Ca(2+) transients in isolated adult rat ventricular myocytes. Vasc. Pharmacol. 40 (4), 213–217.

Huang, J., Upadhyay, U.M., Tamargo, R.J., 2006. Inflammation in stroke and focal cerebral ischemia. Surg. Neurol. 66 (3), 232–245.

Ichikawa, K., Tazawa, S., Hamano, S., Kojima, M., Hiraku, S., 1999. Effect of ozagrel on locomotor and motor coordination after transient cerebral ischemia in experimental animal models. Pharmacology 59 (5), 257–265.

Iwasaki, A., Medzhitov, R., 2004. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 5 (10), 987–995.

Kacimi, R., Giffard, R.G., Yenari, M.A., 2011. Endotoxin-activated microglia injure brain derived endothelial cells via NF-kappaB, JAK-STAT and JNK stress kinase pathways. J. Inflamm. 8, 7 (London, England).

Kao, T.K., Ou, Y.C., Kuo, J.S., Chen, W.Y., Liao, S.L., Wu, C.W., Chen, C.J., Ling, N.N., Zhang, Y.H., Peng, W.H., 2006. Neuroprotection by tetramethylpyrazine against ischemic brain injury in rats. Neurochem. Int. 48 (3), 166–176.

Kariko, K., Weissman, D., Welsh, F.A., 2004. Inhibition of toll-like receptor and cytokine signaling–a unifying theme in ischemic tolerance. J. Cereb. Blood Flow Metab. 24 (11), 1288–1304.

Kawai, T., Akira, S., 2007. Signaling to NF-kappaB by Toll-like receptors. Trends Mol. Med. 13 (11), 460–469.

Khan, M., Elango, C., Ansari, M.A., Singh, I., Singh, A.K., 2007. Caffeic acid phenethyl ester reduces neurovascular inflammation and protects rat brain following transient focal cerebral ischemia. J. Neurochem. 102 (2), 365–377.

Kunz, A., Dirnagl, U., Mergenthaler, P., 2010. Acute pathophysiological processes after ischaemic and traumatic brain injury. Best Pract. Res. Clin. Anaesthesiol. 24 (4), 495–509.

Lakhan, S.E., Kirchgessner, A., Hofer, M., 2009. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. J. Transl. Med. 7, 97.

Lambertsen, K.L., Biber, K., Finsen, B., 2012. Inflammatory cytokines in experimental and human stroke. J. Cereb. Blood Flow Metab. 32 (9), 1677–1698.

Lin, Y.L., Wang, G.J., Huang, C.L., Lee, Y.C., Liao, W.C., Lai, W.L., Lin, Y.J., Huang, N.K., 2009. Ligusticum chuanxiong as a potential neuroprotectant for preventing serum deprivation-induced apoptosis in rat pheochromocytoma cells: functional roles of mitogen-activated protein kinases. J. Ethnopharmacol. 122 (3), 417–423.

Longa, E.Z., Weinstein, P.R., Carlson, S., Cummins, R., 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20 (1), 84–91.

McColl, B.W., Allan, S.M., Rothwell, N.J., 2009. Systemic infection, inflammation and acute ischemic stroke. Neuroscience 158 (3), 1049–1061.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683–765.

Moretti, A., Ferrari, F., Villa, R.F., 2015. Neuroprotection for ischaemic stroke: current status and challenges. Pharmacol. Ther. 146, 23–34.

Nakase, T., Sohl, G., Theis, M., Willecke, K., Naus, C.C., 2004. Increased apoptosis and inflammation after focal brain ischemia in mice lacking connexin43 in astrocytes. Am. J. Pathol. 164 (6), 2067–2075.

Park, J.S., Shin, J.A., Jung, J.S., Hyun, J.W., Van Le, T.K., Kim, D.H., Park, E.M., Kim, H.S., 2012. Anti-inflammatory mechanism of compound K in activated microglia and its neuroprotective effect on experimental stroke in mice. J. Pharmacol. Exp. Ther. 341 (1), 59–67.

Peruzzotti-Jametti, L., Donega, M., Giusto, E., Mallucci, G., Marchetti, B., Pluchino, S., 2014. The role of the immune system in central nervous system plasticity after acute injury. Neuroscience 283, 210–221.

Rakoff-Nahoum, S., Medzhitov, R., 2008. Innate immune recognition of the indigenous microbial flora. Mucosal Immunol. 1 (Suppl. 1), S10–S14.

Shukla, V., Shakya, A.K., Perez-Pinzon, M.A., Dave, K.R., 2017. Cerebral ischemic damage in diabetes: an inflammatory perspective. J. Neuroinflammation 14 (1), 21.

Stamatovic, S.M., Keep, R.F., Kunkel, S.L., Andjelkovic, A.V., 2003. Potential role of MCP-1 in endothelial cell tight junction 'opening': signaling via Rho and Rho kinase. J. Cell Sci. 116 (Pt 22), 4615–4628.

Vila, N., Castillo, J., Davalos, A., Esteve, A., Planas, A.M., Chamorro, A., 2003. Levels of anti-inflammatory cytokines and neurological worsening in acute ischemic stroke. Stroke 34 (3), 671–675.

Wang, Q., Tang, X.N., Yenari, M.A., 2007. The inflammatory response in stroke. J. Neuroimmunol. 184 (1–2), 53–68.

Xiang, B., Xiao, C., Shen, T., Li, X., 2018. Anti-inflammatory effects of anisalcohol on lipopolysaccharide-stimulated BV2 microglia via selective modulation of microglia polarization and down-regulation of NF-kappaB p65 and JNK activation. Mol. Immunol. 95, 39–46.

Xu, S.H., Yin, M.S., Liu, B., Chen, M.L., He, G.W., Zhou, P.P., Cui, Y.J., Yang, D., Wu, Y.L., 2017. Tetramethylpyrazine-2′-O-sodium ferulate attenuates blood-brain barrier disruption and brain oedema after cerebral ischemia/reperfusion. Hum. Exp. Toxicol. 36 (7), 670–680.