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Neuroprotective effects of total steroid saponins on cerebral ischemia injuries in animal model of focal ischemia/reperfusion

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Abstract

Total steroid saponins (TSSN) isolated from *Dioscorea zingiberensis* C.H.Wright (DZW), a unique Traditional Chinese Medicine, is known for its potential usage in various types of diseases. However, there are a little evidences about its neuroprotective effect in transient focal ischemia-reperfusion (I/R) cerebral injury. Therefore, the current study was carried out to investigate the effect of TSSN on neuroprotection and its potential mechanisms in the rat I/R model by middle cerebral artery occlusion (MCAO) for 90 min. The rats were each treated with TSSN (30 mg/kg, 10 mg/kg, and 3 mg/kg) or Nimodipine (20 mg/kg) daily for 6 days before MCAO. Then, the neurological deficit score, cerebral infarct volume, and brain water content were measured at 24 h after reperfusion. Meanwhile, the histopathological changes and AQP-4 protein activities were examined in hippocampal CA1 and the cortex of ipsilateral ischemic cerebral hemisphere by hematoxylin & eosin staining and immunohistochemistry, respectively. The indices of oxidative stress in the serum were also obtained, and NF- κ B and ERK 1/2 protein expressions in the injured brain were evaluated by western blotting. The results indicated that the pre-treatment with these drugs not only significantly reduced cerebral infarct volume, brain water content and improved neurological deficit score, but also restored neuronal morphology and decreased the AQP-4 positive cells in CA1 and the cortex. Moreover, it markedly restored the level of oxidant stress makers (CAT, SOD, MDA, NO and iNOS) to their normal range in serum. In addition, the increased NF- κ B and ERK 1/2 protein expressions were alleviated as compared with the I/R group. These findings demonstrate that TSSN exhibits promising neuroprotection effects against the transient focal ischemia-reperfusion (I/R) cerebral injury in the rat experimental model, where the underlying mechanisms might be mediated through inhibition of anti-edema as well as anti-oxidative effects by inactivation of NF- κ B and ERK 1/2 signalling pathway.

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Conflict of Interest

All authors declare that there are no conflicts of interest in this research.

Keywords

Dioscorea zingiberensis; Dioscoreaceae; steroid saponins; middle cerebral artery occlusion; ischemia-reperfusion; anti-edema; anti-oxidative

Introduction

Cerebral ischemia is a leading cause of morbidity, mortality, and long-term disability worldwide, and it occurs most commonly in stroke or “Brain attack”, which results in significant social, financial and personal problems in our community [1]. Reperfusion plays an important role in curing cerebral ischemic injury. Restoration of blood flow to the ischemic brain as early as possible is a way to rescue the patients. However, reperfusion itself also has the potential risk to produce additional injuries such as irreversible brain damage and neuronal injury in the ischemic brain [2]. Although multiple major pathological mechanisms of cerebral ischemia-reperfusion (I/R), including excitotoxicity, ionic imbalance, oxidative stress, inflammation and apoptosis [3, 4], have been implicated, the pathogenesis of cerebral ischemia/reperfusion (I/R) injury is not yet completely understood until now. Because the cascades of mechanisms are involved in the I/R process, there is no drug available for curing the injuries induced by I/R. Therefore, the development of potential agents having neuroprotective activity with minimum side effects is urgently needed.

The use of herbal medicines, such as *Panax notoginseng* [5], *Scutellaria baicalensis* Georgi [6], *Puerariae* [7], and *Nardostachys* [8], for the treatment of cerebral ischemia has a long history in China. *Dioscorea zingiberensis* C.H.Wright (DZW), a unique plant growing in China, has been used as a folk treatment for cough, anthrax, rheumatoid arthritis, tumefaction, sprain [9] as well as various cardiac diseases in TCM for a long time. Total steroid saponins (TSSN) isolated from the rhizomes of DZW were reported to be the biologically active constituents responsible for the broad physiological and pharmacological functions of this medicine. In the past extensive studies have demonstrated its various medicinal effects including curing cardiac diseases through enhancing the coronary blood flow, protecting cardiac muscles, improving peripheral circulation, inhibiting platelet aggregation, decreasing cholesterol and triglyceride, and relieving angina pectoris [10]. However, to our knowledge, there are little research reports on neuroprotective effects of the TSSN from DZW against I/R.

Therefore, the primary objective of this study was designed to evaluate whether the TSSN isolated from DZW has the neuroprotective effect against transient focal cerebral ischemia-reperfusion injury. The biochemical and histological parameters, such as infarct volume, neurological deficit score, anti-oxidant, and anti-edema, were systematically investigated via this entire experiment in the rat model of middle cerebral artery occlusion (MCAO).

Results

The prominent neurological deficit scores, cerebral infarct size and degree of brain edema in the I/R group were all observed by MCAO-induced ischemia for 90 min after 24 h

reperfusion and compared with the sham-operated group ($p<0.01$ for neurological deficit scores, $p<0.01$ for cerebral infarct size, and $p<0.05$ for brain water content. Fig. 1). Pre-treatment with Nimodipine and TSSN attenuated the severities in these three indicators induced by I/R, all of which were still higher than that in the sham-operated group. There was no significant difference in these symptoms and brain water content between the 3 mg/kg and 10 mg/kg administration groups. However, there was significant improvement in the 30 mg/kg group ($p<0.05$ for brain water content, $p<0.01$ for other two), which had almost the equally significant difference to the Nimodipine group.

It revealed that there were normal neuronal structures in both CA1 and the cortex in the sham-operated group (Fig. 2), whereas the I/R group showed shrunken neurons and swelling neurons in ischemic regions. In addition, some neurons were injured and lost after cerebral ischemia. However, many survived neurons were observed in the TSSN (30 and 10 mg/kg) and Nimodipine groups.

The anti-edema effects of TSSN were examined by the expression of AQP-4, a member of the aquaporin family of integral membrane proteins, using immunohistochemical method (Fig. 3). The sham-operated group demonstrated little positive AQP-4 staining in the ipsilateral hippocampus CA1 and the cortex of the injured brain, suggesting low AQP-4 expression in these areas (Fig. 3 A and B). In the I/R group, however, strongly positive staining of AQP-4 was observed, suggesting marked edema. After TSSN and Nimodipine treatment, AQP-4 staining was significantly reduced as compared with the I/R group, indicating that I/R-induced edema was relieved.

To illustrate the effects of TSSN on the oxidative stress induced by global cerebral I/R, MDA and NO contents, CAT, SOD, and iNOS enzyme activities were measured after 24 h of I/R injury. The results showed that CAT and SOD enzyme activities were significantly decreased in the I/R group compared with the sham-operated group ($p<0.01$ for CAT, $p<0.01$ for SOD, respectively. Fig. 4). Pre-treatment of rats with TSSN and Nimodipine obviously restored their activities in a dose-depend manner. Conversely, a significant increase in NO and MDA contents with iNOS activity was all found in the I/R group compared with the sham-operated group ($p<0.01$ for NO, $P<0.05$ for iNOS, $P<0.01$ for MDA, respectively). The pre-treatment of TSSN and Nimodipine efficiently prevented the formation of NO and MDA, and enhancement of iNOS. Among the three TSSN treatment groups, there was no significant effect in these five biochemical indicators under the 3 mg/kg, and so was with 10 mg/kg in MDA content and iNOS enzyme. The 10 mg/kg and 30 mg/kg TSSN groups had much greater effect than the 3 mg/kg dose on elevating the CAT and SOD and suppressing NO, MDA, and iNOS after I/R, especially in the dose of 30 mg/kg which had almost the same effects produced from Nimodipine.

There was a significant increase in NF- κ B and ERK 1/2 expression ($p<0.01$ for NF- κ B, $p<0.01$ for ERK 1/2. Fig. 5) in the ischemic hemisphere of rats injured by MCAO-induced for 90 min and reperfusion for 24 h (I/R) compared with the sham-operated group. After pre-treatment with TSSN or Nimodipine, both expressions were markedly decreased compared with the I/R group in a dose-depend manner. There were significant differences in the ERK 1/2 expression for the three doses of TSSN (3 mg/kg, 10 mg/kg, and 30 mg/kg).

The 3 mg/kg group showed no significant effect in the NF- κ B expression. What's more, the dose of 30 mg/kg and 10 mg/kg produced the better effect than the Nimodipine, and these two protein expressions in the 30 mg/kg group ($p < 0.01$ for NF- κ B and ERK 1/2 vs. I/R) had almost the same level with the sham-operated group.

Discussion

In the present study, we evaluated for the first time the effects of TSSN from DZW in rat model of transient focal cerebral ischemia by MCAO method, a reliable and less invasive stroke model of temporary regional ischemia. The toxicity experiment of TSSN in rats was carried out prior to the assessment of its anti-ischemia effect. In this test, death, abnormal behavioural changes, or toxic symptoms were not observed on rats at doses from 200 mg/kg to 3000 mg/kg at two-week period, suggesting that TSSN had no toxic in vivo at the above doses and the approximate lethal dose was beyond the 3000 mg/kg. In preliminary experiment, 60 mg/kg, 1/50th of dose, was adopted as the high dose, while a half and quarter of it as the middle and low doses, respectively. They displayed potential anti-ischemia effect but with unsatisfactory dose-dependent results. Finally, three doses described in the method section were used in the present experiment. The MCAO by Longa's method with minor modification [11] was used to establish the ischemia and reperfusion model. When the round tip of suture reached the proximal segment of the anterior cerebral artery, most sources of blood that flowed into the cortex and basal ganglia were occluded. If this procedure was strictly operated, the blood flow was within the ischemic range. In order to guarantee the success of this model, the neurological deficit score was measured immediately after this surgical work. The rats with no obvious ischemic symptoms were deleted from the group.

Here, it demonstrated that pre-treatment with TSSN attenuated the cerebral injury as evidenced by the improvement of neurological deficit scores, reduction of cerebral infarct size and brain edema induced by a transient focal cerebral I/R in rats. What's more, the mortality was recorded at the same time. There was no death in sham-operated and 30 mg/kg groups after ischemia for 90 min followed by reperfusion for 24 h (0 death rate) whereas death occurred in 5 of 15 rats in I/R group (33.3% death rate), 2 of 12 rats in Nimodipine group (16.7% death rate), 1 of 11 rats in 10 and 3 mg/kg groups (9.1% death rate). It further confirmed that the TSSN indeed revealed the potentially anti-ischemia effect with this director indicator. Brain edema is a commonly observed pathophysiological change in I/R. During the damage of I/R, the blood brain barrier (BBB) is destroyed and the permeability of blood vessel becomes weak due to release of massive vasoactive substances, finally leading to edema in the ischemic brain. AQP-4 is a member of the AQP family, which plays an important role in water transport, and widely expressed in the nervous system [12]. This AQP-4 expression was significantly increased in the hippocampal CA1 and cortex observed by the immunohistochemistry method after I/R in the current study. The TSSN pre-treatment could remarkably decrease its expression, indicating that TSSN could exert anti-edema effects against I/R.

Overwhelming evidences have accumulated implying that excessive reactive oxygen species (ROS) and oxidative stress play key roles in the pathophysiology of I/R injury [13]. Normally, there is a balance between the formation of ROS and its consumption by the

endogenous protective systems. During the I/R, sudden burst of ROS, which causes oxidative damage to lipids, proteins, and DNA, cannot be handled by the endogenous anti-oxidant systems because of low activities of anti-oxidative enzymes [14]. Consequently, the brain is exposed to the high level of ROS leading to cerebral dysfunction and cell death. The SOD and CAT activities were markedly decreased with increased MDA content in the I/R group. Pre-treatment with Nimodipine and TSSN for 7 days could significantly restore these enzyme activities and MDA content in a dose-dependent manner. The promising results suggested that TSSN could protect the brain from I/R through anti-oxidant stress mechanism. Our previous phytochemical investigation revealed that there were various types of steroid saponins in the TSSN. It is generally known that the steroid saponins consist of two main parts, i.e. aglycone and different sugar moieties attached to it. We inferred that the TSSN could attenuate the oxidant stress produced in I/R probably because of the hydroxyls in the steroid-like compound structures. Another free radical that is elevated in cerebral ischemia is NO molecule, however, its precise role in this neuropathology remains debatable [15]. There are three subtypes of NOS namely eNOS nNOS and iNOS [16]. It is reported that the synthetic NO catalyzed by eNOS during the I/R could protect the neuron, while the NO catalyzed by iNOS and nNOS shows obvious neurotoxicity by generating free radicals [17]. In the present study, the results of NO content and iNOS activity were notably reduced with pre-treatment with TSSN compared with the I/R group. As reported before [18], the diosgenin a kind of aglycone could suppress the iNOS expression. There are many spirostane steroid saponin compounds in TSSN with the aglycone of diosgenin such as dioscin and gracillin, and these compounds are possibly the potentially anti-ischemia ingredients after losing the sugar moieties.

NF- κ B, a key transcription factor, is involved in many events during the pathophysiologic process of cerebral ischemia and reperfusion such as inflammatory, excitotoxic, and oxidative responses [19]. In normal physiological conditions, the NF- κ B is located in the cytoplasm, bound to inhibitory binding proteins I κ B, and remains as inactive forms [20]. When the cells are stimulated by various external stimuli such as the cerebral ischemia, the I κ B is phosphorylated and degraded. Then, it is released from the NF- κ B p65-p50 dimer, finally leading to activation of NF- κ B signalling pathway [21]. The dissociative NF- κ B translocates to the nucleus, binds to κ B binding sites on DNA, and regulates transcriptional activation of the target genes like cytokines. These products cause the secondary injury in cells. According to the results of western blotting analysis, there was a significant increase in NF- κ B protein expression in ischemic cerebral hemisphere in I/R rats compared to the sham-operated group. This increase was alleviated by the TSSN pre-treatment, indicating that the inhibition of the activation of NF- κ B might be another neuroprotective mechanism of TSSN through suppressing the translocation of NF- κ B from cytoplasm to nucleus. However, the accurate mechanism whether the TSSN inhibited the phosphorylation of I κ B, or prevented the dissociation of complex of I κ B-p65-p50, should be further study.

Extracellular signal-regulating kinases 1 and 2 (Erk1/2), the best-characterized and prototype members of the mitogen-activated protein kinase (MAPK) signal pathway superfamily, are thought to be an early indicator for the development of ischemia reperfusion [22]. After the cell is subjected to harmful stimuli, the Erk1/2 is phosphorylated at conserved threonine and tyrosine residues, and then activated. Although studying for

many years, the precise role of ERK1/2 pathway in cerebral ischemia and reperfusion injury has remained controversial [23]. Most studies have verified it as an important protective signal pathway in the process of initiating neuronal damage [24], while others consider it as a supporter for neuronal survival [25]. The elevated p-Erk 1/2 in the ischemic brain after 24 h reperfusion in this study might promote inflammatory mediators and oxidative stress. These mediators could regulate the release of cytokines and free radicals, and then these harmful products deteriorate the ischemic brain with a cascade reaction. The increase or these mediators was attenuated by the pre-treatment of rats with TSSN, which implied that the neuroprotection of TSSN probably inactivated the Erk 1/2 signalling pathway through suppressing the phosphorylation of Erk 1/2. But, the exact mechanism of how TSSN prevents the phosphorylation of Erk 1/2 should be further explored.

In summary, TSSN isolated from DZW shows robust neuroprotective effects in rats exposed to transient focal cerebral I/R. This protective mechanisms may be involved via anti-oxidative and anti-edema actions mediated by activation of NF- κ B and ERK 1/2 pathways.

Materials and Methods

Animals and chemicals

Healthy adult male Sprague-Dawley rats aged 8-10 weeks (weight: 270~320 g) used in this experiment were purchased from the Experimental Animal Center of The Fourth Military Medical University (Shaanxi, China). The animals were housed in standard polypropylene cages (5 rats/cage) under a normal 12 h:12 h light/dark cycle at room temperature of $24 \pm 1^\circ\text{C}$ and relative humidity of 55-65%. They were allowed at least one week to adapt to the laboratory environment before surgery with free access to tap water and food pellets. All studies were carried out in accordance with the Animal Experimental Committee of the Fourth Military Medical University (approved on May 20, 2013, NO. FMMU 2013-05) and local Animal Ethical Committee (approved on January 1, 2012, NO. 3245/2012) for minimizing animal suffering.

The rhizomes of *Dioscorea zingiberensis* C.H.Wright were provided by Yangtze River Pharmaceutical Industry Co., Ltd (Jiangsu, China) and identified by Professor Yazhou Wang (Northwest University, Xi'an 710069, China). Its voucher specimens (HJ20100925-10) were deposited in the Biology and Medicine Key Laboratory of Shaanxi province, China. The Nimodipine pills (BCP0007538, 98% purity) were obtained from Zhejiang Yixing Pharmaceutical Co., LTD (Lanxi, Zhejiang Province, PRC). TTC reagent namely 2, 3, 5,-triphenyltertazolium chloride (1014795, 98% purity) was purchased from Amresco and dissolved in PBS. The inflammatory factor ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, PRC). AQP-4 primary antibody (rabbit anti-rat) was supplied from Wuhan Boster Biological Technology Co., LTD (Wuhan, Hubei Province, PRC), while the other antibodies from Beijing Zhongshan Golden Bridge Biotechnology Co., LTD (Beijing, PRC). All other chemicals and solvents were of analytical grade.

Preparation of total steroid saponins extract

Dried raw rhizomes of *Dioscorea zingiberensis* C.H.Wright (3.5 Kg) were powdered and extracted with 70% ethanol for three times. The ethanol solution was combined and evaporated to dryness under reduced pressure by a rotary evaporator, and the residue was redissolved in water, and subjected to centrifugation. The supernatant was separated on a D-101 macroporous resin column (0.15 m × 1.0 m, 5.0 Kg macroporous resin), by eluting with 60% ethanol. The effluent was concentrated under reduced pressure. The syrup thus obtained was dissolved in 1 L water, and extracted with an equal volume of n-butanol six times successively. The pooled n-butanol solution was concentrated to obtain 140 g of residue for subsequent experimental use. The chemical profiles of the same batch extract (10041511) which was ready to use in this experiment have been published previously [26].

Drug administration and induce of MACO model

Animals were randomly allocated into six groups with 20 rats in each group: sham-operated, I/R model, Nimodipine-treated, and TSSN-treated with three different dose groups. Nimodipine and TSSN were administered orally once a day for seven days before MCAO at doses of 20 mg/kg, 30 mg/kg, 10 mg/kg, and 3 mg/kg, respectively, while sham-operated and MACO groups were given the equal volume of saline. Half an hour after the sixth administration, the operation was performed. The animals were subjected to transient middle cerebral artery occlusion (MCAO) model using the intraluminal thread [11]. Briefly, rats were anesthetized with 10% chloral hydrate (0.35 g/kg) intraperitoneally (i.p). The left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed and carefully isolated from the vagus nerve under sterile conditions. The pterygopalatine branch of ICA was sealed with electric coagulator. The distal end of the ECA was ligated with a 4-0 silk suture. A 25-mm length of nylon filament (Ø 0.24-0.26 mm), with its tip dipped in heparin before use, was introduced into the ECA lumen through a small puncture. The nylon filament was gently advanced from the ECA into the lumen of the ICA, a distance of 18-20 mm (as measured from the carotid bifurcation), until the tip of the filament blocked the origin of the middle cerebral artery (MCA). After 90 min ischemia, the nylon filament was withdrawn to establish reperfusion. The same surgical procedure was applied on sham-operated rats, but without occlusion of the middle cerebral artery. During surgical procedure, the body temperature of rats was maintained and monitored at $37 \pm 0.5^{\circ}\text{C}$ with an infrared heat lamp and a heating pad.

Measurement of neurological deficit score, cerebral infarction, and brain edema

The neurological deficit score of each rat, according to Longa's method of a 5-point scale [11] with minor modifications (no neurological deficit=0; failure to extend right paw fully=1; circling to right=2; falling to right=3; being unable to walk spontaneously and depression of consciousness=4), was evaluated by the same experimenter, who was blinded to the different treatments in the experiment, 24 h after reperfusion.

Rats were sacrificed with an overdose of chloral hydrate after evaluation of the neurological deficit score. Brain tissues were immediately removed and weighed to obtain brain tissue wet weight. Then, they were placed in the refrigerator under -20°C for about 20 min, cut into five coronal slices continuously from front to back with a blade, and immersed into 4% TTC

at 37°C in 0.2 M tris buffer (pH 7.4) for 30 min in the dark. The brain slices were turned over for several times, so that they could be evenly exposed to the dye. The TTC stained sections, where the viable cerebral tissue was stained red while the infarct cerebral tissue remained pale, were photographed with a digital camera and the infarct area of each section was measured using Image-Pro plus (version 6.0) analyzer software. The infarct size was expressed as a percentage fraction of total global brain [27].

The brain edema was determined by evaluating the brain water content according to the wet-dry method [28]. In brief, after measurement of infarct volume, all of the samples including both infarct and non-infarct sections were dried in an oven at 110°C for 24 h and weighed again to obtain the dry weight. Then, brain edema was calculated as follows: brain water content (%) = [(wet weight-dry weight)/wet weight] × 100.

Brain tissues preparation

After establishing the I/R model, there are mainly two regions in brain tissue, i.e. ischemic core and penumbra. They were separately dissected according to the diagram (Fig. 1). In this study, the evaluation was mainly performed on the penumbra section. For histopathological examination and immunohistochemical analysis, rats under anesthesia were transcardially perfused with heparinized saline at first, followed by 4% paraformaldehyde (PF) in 0.1 mol/L phosphate buffer (pH=7.4) after 24 h reperfusion. After the rats were sacrificed the right brain was gently removed and post-fixed in the same fixative (4% PF) overnight at 4°C. After dehydration, the brain tissues were embedded in paraffin. A coronal section from posterior to the optic chiasm in each tissue was obtained, and the serial paraffin-embedded 5 µm thickness sections were subjected to the subsequent analysis. For western blotting, the ischemic brains were immediately removed and frozen in liquid nitrogen and stored at -80°C until use.

H&E staining immunohistochemistry assessment

After deparaffinization and hydration with a graded alcohol series, sections obtained in 2.5. were stained with hematoxylin-eosin (H&E) for histological evaluation. The treated histological sections were washed thrice in PBS (pH 7.4) and immersed in 0.5% hydrogen peroxide for 10 min, then washed thrice with PBS again. After blocking in normal goat serum for 20 min, sections were successively incubated with primary antibodies against AQP-4 overnight at 4°C. Next day the sections were washed thrice with PBS and incubated with secondary antibody (goat-anti-rabbit polyclonal antibody) for 40 min at 37°C. The sections were again washed thrice with PBS and then incubated for 30 min with Streptavidin-Horseradish peroxidase that can conjugate with the secondary antibody. Thereafter, sections were washed thrice with PBS, incubated with 3, 3-di-aminobenzidine (DAB) as a chromogen for light microscopy, for 3-5 min, and stopped the reaction by washing in PBS. The slides were then dehydrated, dried, and covered with a cover slip. All incubations were performed in a humidified chamber. Negative control sections from each animal received identical staining, except that the primary antibody was omitted. Finally, the immunopositive cells in ischemic hippocampus CA1 and the cortex of brains were examined under an optical microscope. The number of positive cells was counted by an investigator who was blinded to the experimental condition.

Western blotting analysis for NF- κ B and ERK 1/2 protein

The prepared brain tissue in 2.5. was homogenized by adding to a 1:5 tissue weight of protein extraction buffer in a glass homogenizer. The protein concentration of samples was determined using a BCA protein assay kit. Homogenate samples (50 μ g), mixed with an equal volume of a sample buffer and heated at 95°C for 5 min, were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membrane was blocked with PBST containing 5% non-fat dry milk for 1 h, and then incubated in the corresponding primary antibodies, NF- κ B and ERK 1/2, for 1 h at room temperature. After washing three times with TBST, the membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, diluted in the blocking buffer (dilution: 1:1000) for 1 h, and washed again three times in PBST buffer.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), of which protein expression levels were normalized, was used as a loading control. The protein band of interest was visualized using an ECL chemiluminescence system (ECL plus; Amersham Biosciences, NJ, USA) and the density of each band was quantified using an image analysis software (AlphaEase FC).

Estimation of oxidant stress system (CAT, SOD, MDA, NO and iNOS) concentrations in rat serum

Rats were anaesthetized with chloral hydrate intraperitoneally (i.p) and sacrificed by withdrawing the blood from the abdominal vein after 24 h reperfusion. The blood sample was centrifuged at 3000 rpm for 20 min to obtain serum and stored at -20°C prior to analysis. Levels of MDA and NO as well as the activities of CAT, SOD, and iNOS in the serum were investigated by commercially available corresponding kits. All the procedures of the used kits were performed according to the manufacturer's instructions. The results were expressed in U unit per millilitre (nmol per millilitre for MDA, μ mol per millilitre for NO).

Statistical analysis

All data were analyzed with SPSS software (version 19.0) and expressed as mean $\pm$ SD (standard deviation). Statistical evaluation was performed using one-way analysis of variance (ANOVA) followed by Student's t-test to compare the significance in difference between the treated groups and the control groups. A value of $P < 0.05$ or $P < 0.01$ was considered to be statistically significant.

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Figure 1. The diagram of boundary of ischemic penumbra and core region and the protective effect of TSSN and Nimodipine in rats at 24 h after MCAO

(a) Diagram showing the ischemic brain tissues corresponding to ischemic penumbra and core. In this experiment, the assessment was evaluated on penumbra section after separated. (b) Representative brain sections stained with TTC. Red colored regions indicate non-ischemia and pale colored regions indicate ischemic portion of brain. (c) Neurological deficit scores. (d) Infarct volume. (e) Brain water content. All data were expressed as mean $\pm$ SD (n=10 per group). [#]p versus the sham-operated group and ^{*}p versus the I/R group.

Figure 2. The histological changes in the rat brain subjected to MACO by 90 min at 24 h were evaluated by H&E staining
(A) CA1. (B) Cortex. The original magnification was 400 × and scale bars represent 50 μm.

Figure 3. Expression of AQP-4 in hippocampal cornu ammonis 1 and cortex of ischemic hemisphere brain after MCAO for 90 min followed by reperfusion for 24 h with immunohistochemical analysis assay

(A) CA1. (B) Cortex. Photomicrographs are x 400 (scale bar, 50 μ m).

Figure 4. The effect of TSSN and Nimodipine on oxidant stress makers in the serum of rats subjected to MCAO at 24 h after reperfusion

(a) NO levels, CAT and SOD activities. (b) iNOS activities and MDA levels. All data were expressed as mean $\pm$ SD (n=10 per group). #p versus the sham-operated group and *p versus the I/R group.

Figure 5. The effects of TSSN and Nimodipine pre-treatment on expressions of ERK 1/2 and NF-κB protein in the ischemic brain of the rats exposed to 90 min MCAO-induced ischemia and 24 h reperfusion

(a) Representative results of Western blot. (b) Quantitative analysis. The protein levels were normalized against the GAPDH, which served as the loading control. Values are expressed in the relative optical density and represent as means $\pm$ SD (n=3 per group). [#]p versus the sham-operated group and ^{*}p versus the I/R group.