



Xuesaitong injection (lyophilized) combined with aspirin and clopidogrel protect against focal cerebral ischemic/reperfusion injury in rats by suppressing oxidative stress and inflammation and regulating the NOX2/IL-6/STAT3 pathway

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Background: Combination of aspirin (ASA) and clopidogrel (CLP) [dual antiplatelet therapy (DAPT)] has been limited in reducing early recurrent stroke events. Xuesaitong injection (lyophilized) (XST) made of total saponins from *P. notoginseng*, which significantly improves cerebral circulation and has been widely used in clinical applications for decades to treat and prevent ischemic stroke. Here, we confirmed the protective effect and mechanism of XST combined with DAPT (XST+ASA+CLP) on cerebral ischemia/reperfusion injury, exploring their better pharmacological action for clinical patients.

Methods: Sprague-Dawley rats (SD rats) (n=9 in each group) were randomly assigned to three groups and pretreated with XST, ASA+CLP, or XST+ASA+CLP for 7 days. Then rats were subjected to 2 h of middle cerebral artery occlusion (MCAO) followed by reperfusion for 24 h. Therapeutic effect of XST+ASA+CLP was measured by infarct volume, neurological behavior and regional cerebral blood flow (rCBF). Inhibition of neuronal apoptosis and glial cells was determined by immunofluorescent staining. We studied the protein levels of neurotrophic factors, neuroplasticity-related factors, oxidative stress indicators and inflammatory factors by ELISA assay.

Results: XST+ASA+CLP group showed significant reduction in infarct volumes and neurological deficit scores. XST+ASA+CLP group also had higher levels in rCBF and synaptic growth, and showed remarkable inhibition of microglia and astrocytes activation and the neuronal apoptosis. In addition, XST+ASA+CLP group had lower levels of NADPH, protein carbonyl, 4-hydroxynonenal (4-HNE), 8-hydroxydeoxyguanosine (8-OHdG) and several inflammatory cytokines. Moreover, XST+ASA+CLP group also had lower levels of NOX2, inducible nitric oxide synthase (iNOS), interleukin (IL)-6, and p-STAT3/STAT3.

Conclusions: These results demonstrate that a combination of XST, ASA, and CLP effectively protected rats against middle cerebral artery occlusion/reperfusion (MCAO/R) injury by suppressing the NOX2/IL-6/STAT3 pathway. These novel findings provide theoretical basis and experimental evidence for the rationality of clinical combined use of drugs in the treatment of ischemic stroke.

Keywords: Xuesaitong injection; aspirin (ASA); clopidogrel (CLP); oxidative stress; inflammation

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Introduction

Stroke is a cerebrovascular disease that causes irreversible neurological damage and remains one of the leading causes of death and disability in adults worldwide (1). Various pharmacological agents, which target neuronal death blockade or neuronal survival and regeneration enhancement after stroke, have been the bases of neuroprotection research (2). Therapeutics targeting neuronal death blockade are currently identified as the basic way to overcome the limitations of stroke treatments. However, effective antagonists that treat stroke are very limited in clinical and basic research.

Multiple mechanisms, including excitotoxicity, inflammation, oxidative stress, apoptosis, calcium influx, and mitochondria injury, are key contributors to brain damage after stroke (2). Inflammation caused by stroke may contribute to neural dysfunction, cell death, and poor clinical outcomes (3). As a resident immune cell of the central nervous system (CNS), phagocytic microglia maintain immune homeostasis primarily through regulated pro- and anti-inflammatory signaling pathways (4). Interestingly, ischemic stroke increases the production of early pro-inflammatory cytokines, leading to deep immunosuppression and bilateral peripheral inflammatory responses (5). Furthermore, ischemic tissue reperfusion aggravates cerebral injury caused by oxidative stress, which generated excessive reactive oxygen species (ROS) (6). The role of ROS sources should be clarified because of their involvement in oxidative stress. Nicotinamide adenosine dinucleotide phosphate (NADPH) oxidases are the major sources of ROS in the pathological process of cerebral ischemia/reperfusion (I/R) injury (7,8). NADPH oxidase (NOX) is a multiunit enzyme originally discovered in neutrophils and has recently been proved to be a major producer of ROS and oxidative stress (9). Our *in vitro* study showed that notoginsenoside R1 (NG-R1) exhibited neuroprotection against cerebral I/R injury by inhibiting NOX activity via endoplasmic reticulum (ER)-dependent activation of the Akt/Nrf2 pathway (10). At present, five NOX enzyme isoforms (NOX 1–5) have been identified and localized. NOX2, now known as gp91phox, and NOX4, are primarily localized in the cerebral cortex and hippocampus

CA1 (11,12). The infarct sizes significantly decreased in NOX2 inhibitor-treated animals and NOX2 knockout animals (13,14), therefore NOX2 is extremely important in oxidative stress-induced neuron damage following stroke. However, whether NOX inhibition reduces neuroinflammation and improves neurological function in ischemic stroke is largely undefined.

Aspirin (ASA) is a prophylactic antiplatelet drug for people with cardiovascular and cerebrovascular diseases, such as heart attack and stroke, in high-risk populations with thrombosis. The antithrombotic action of ASA works by preventing the biosynthesis of thromboxane-A₂ (TXA₂) by inactivating its reversible COX-1, which inhibits platelet aggregation (15). Clopidogrel (CLP) is an oral antiplatelet agent used to inhibit thrombosis in coronary artery disease, cerebrovascular disease, and peripheral vascular disease. The anti-aggregation activity of CLP is attributed to short-acting metabolites that are produced by the cytochrome P450-dependent pathway in the liver, which prevents the binding of adenosine diphosphate (ADP) to its platelet receptor (16). Long-term use of antiplatelet drugs in patients with noncardiac embolic stroke or transient ischemic attack, are crucial for extended secondary prevention of stroke and other cardiovascular events; such drugs include ASA monotherapy, CLP monotherapy and combination of ASA and extended-release dipyridamole treatment (17). Moreover, the American Heart Association and the American Stroke Association have issued updated clinical practice guidelines on dual antiplatelet therapy (DAPT). Based on these guidelines, DAPT with ASA and CLP provided additional benefits in reducing the occurrence of numerous vascular events and inhibiting intense platelet aggregation in patients with early recurrent stroke and coronary artery disease (18–20). However, due to the ceiling effect and unresolved bleeding risk during the treatment process, DAPT therapy is in trouble.

At present, Chinese patent medicines for treating acute ischemic stroke are mainly drugs for promoting blood circulation and removing blood stasis. *Panax notoginseng*, also known as Sanqi, is an ancient Chinese medicinal plant that has great clinical value to regulate cardiovascular (21) and neurological diseases (22) in China. Xuesaitong

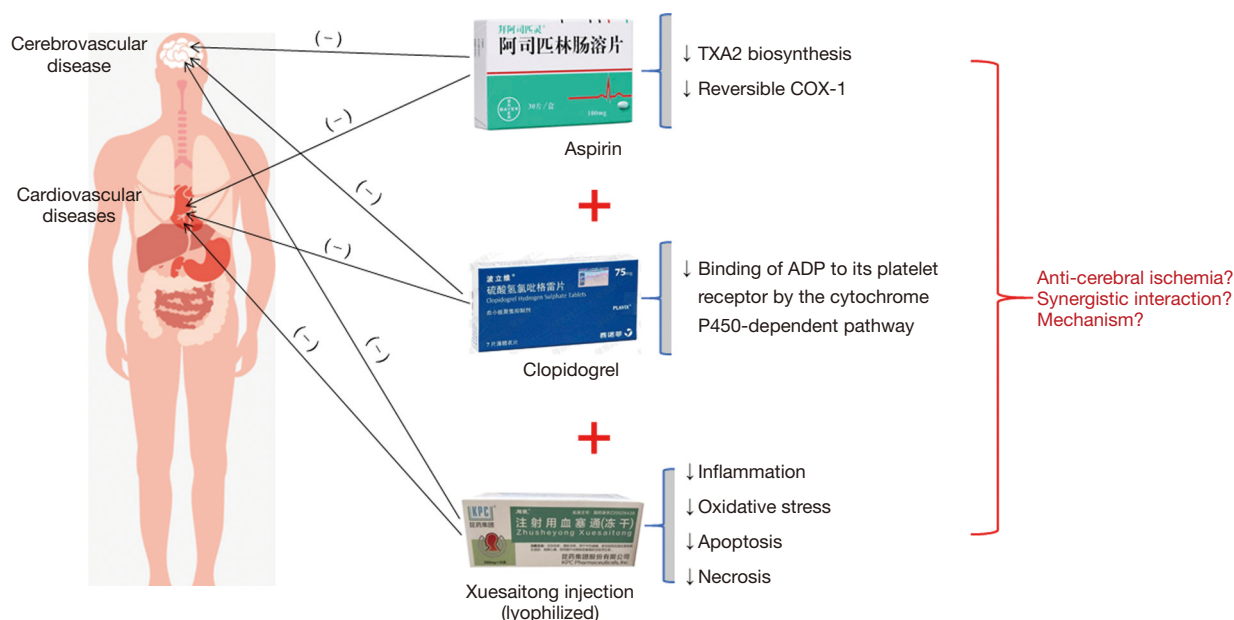


Figure 1 Summary of the pharmacological effects and mechanisms of ASA, CLP and XST. (–) (↓), inactivation or inhibition. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel.

injection (lyophilized) (XST) made of total saponins from *P. notoginseng*, which significantly improves cerebral circulation and has been widely used in clinical applications for decades to treat and prevent ischemic stroke. In the past few decades, more than ten million patients have been cured by injection of XST. Our previous study showed that Xuesaitong capsules combined with ASA attenuates brain dysfunction in rats with ischemic stroke by inhibiting oxidative stress and inflammatory response (23). Other studies have also demonstrated that panax notoginsenoside (PNS) reduces the neurological deficit and cerebral infarct volume and protects the nervous system from I/R injury by inhibiting inflammation, oxidative stress, cell apoptosis, and necrosis (24,25). However, whether the combination of DAPT and XST are superior to XST monotherapy for ischemic stroke remains largely unknown. In the present study, we first investigated the effect of combination of XST+ASA+CLP (ASA 10 mg/kg, CLP 7.5 mg/kg plus XST 40 mg/kg) against inflammation and oxidative stress through the downstream pathway, NOX2/IL-6/STAT3, after ischemic stroke. These benefits provide experimental evidence for clinical precise positioning and rational drug use (Figure 1). We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/apm-20-1681>).

Methods

Animals

Male Sprague-Dawley (SD) rats weighing 300 ± 10 g were purchased from Beijing Vital River Laboratories and used in this study. Five rats were housed per cage on a 12-h artificial light and given free access to food and sterilized drinking water in an animal facility with controlled temperature ($20\text{--}25^\circ\text{C}$) and humidity ($30\text{--}50\%$). All animal care and experimental procedures were reported in accordance with the Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences & Peking Union Medical College and complied with NIH Guidelines for the Care and Use of Laboratory Animals (approval number: SYXK 2017-0020). All efforts were followed to reduce the number of animals used and ensure minimal suffering.

MCAO surgery and regional cerebral blood flow (rCBF) measurement

SD rats were anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injections. Middle cerebral artery occlusion/reperfusion (MCAO/R) was proceeded by experimenters who were unaware of the grouping scheme, following a previously described method (10).

Rats in the sham group were operated using the same method but with no occlusion in middle cerebral artery. The body temperature of rats was maintained at 37 ± 0.5 °C during the MCAO procedure with a heating pad (Sunbeam, USA). The incision was sewed after ischemia and cerebral blood flow in the ischemic core region and peripheral region after reperfusion 24 h was assessed by the laser Doppler flowmeter (moor FLPI-2, UK).

Drug treatment

XST was provided by Kunmingshenghuo Pharmaceutical Co., Ltd.; ASA was obtained from Bayer Medical and Health Co., Ltd.; and CLP was purchased from Sanofi Pharmaceutical Co., Ltd. (Hangzhou, China). Prior to administration, the drugs were freshly prepared in normal saline. Male SD rats were randomly assigned to five groups, which were pre-treated for 7 days before MCAO/R. The sham and MCAO/R groups were given normal saline solution injection intravenously (i.v.) daily. The XST group was treated with XST at a dose of 40 mg/kg i.v. through the tail once a day. The ASA and CLP co-treatment groups (ASA+CLP) were treated with ASA at a dose of 10 mg/kg intragastrically (i.g.) and followed by CLP at a dose of 7.5 mg/kg i.g. every day. The XST, ASA, and CLP co-treatment groups (XST+ASA+CLP) were treated with 40 mg/kg XST i.v., 10 mg/kg ASA i.g., and 7.5 mg/kg CLP i.g. After 7 days, serum, hippocampus, and cortex tissues were collected for mechanism research underlying ischemic stroke in XST+ASA+CLP intervention in rats with MCAO/R.

Neurological score

Neurological performances of all animals were evaluated by two blinded investigators using Zea Longa Scores following a previously described method (10). The neurological function was scored according to a series of scales from 0 to 4. The highest score represents the most severe neurological deficits.

Triphenyltetrazolium chloride (TTC) staining

TTC staining was conducted 24 h post-stroke based on the methods described previously (n=3 for each group) (10,26). Cerebral infarct area was quantified by an image analysis system (Image-Pro Plus 5.0). The infarct volume can be obtained by multiplying the total infarct area by the thickness of the brain sections. Calculating the corrected

infarct volume is contribute to compensate for the error caused by brain edema (10).

Western blot analysis

Western blot method was conducted as previously reported (10). The primary antibodies used in the experiment were as follows: NOX2 antibody (Abcam, USA), inducible nitric oxide synthase (iNOS) antibody (Cell Signaling Technology, USA), anti-IL-6 (Abcam, USA), anti-STAT3 (Abcam, USA), and anti-phospho-STAT3 (Abcam, USA). Blot densities were calculated by ImageJ software.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits (Boster Biotechnology, China) were used according to the manufacturer's instructions to quantify the expression of the oxidative stress markers NADPH, protein carbonyl, 4-hydroxynonenal (4-HNE), 8-hydroxydeoxyguanosine (8-OHdG), and pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, ICAM-1, CD11a, MCP-1, IL-10, and IL-4. In brief, serum and homogenates of the right cortex and hippocampus were prepared for analysis. Oxidative stress markers and inflammatory cytokine levels were expressed as a percentage of the control.

Immunofluorescent staining

Immunofluorescence staining was implemented as previously reported (27). Primary antibodies, namely rabbit monoclonal NeuN antibody, rabbit monoclonal GFAP antibody, and mouse monoclonal Iba-1 antibody, were purchased from Abcam, USA for the experiment. Images were obtained through at least three random visual fields from three separate sections of each sample by using fluorescence microscope (Carl Zeiss, Germany) at magnification of 200 \times . Mean values of Iba-1 and GFAP were calculated for statistical analysis.

TUNEL staining

Neuronal apoptosis in brain tissue was evaluated by TUNEL staining with the neural marker, NeuN (Abcam, USA) using a TUNEL kit (Beyotime Institute of Biotechnology, China) as previously reported (26). The images of TUNEL positive neuronal cells were captured using a fluorescence microscope (Carl Zeiss, Germany).

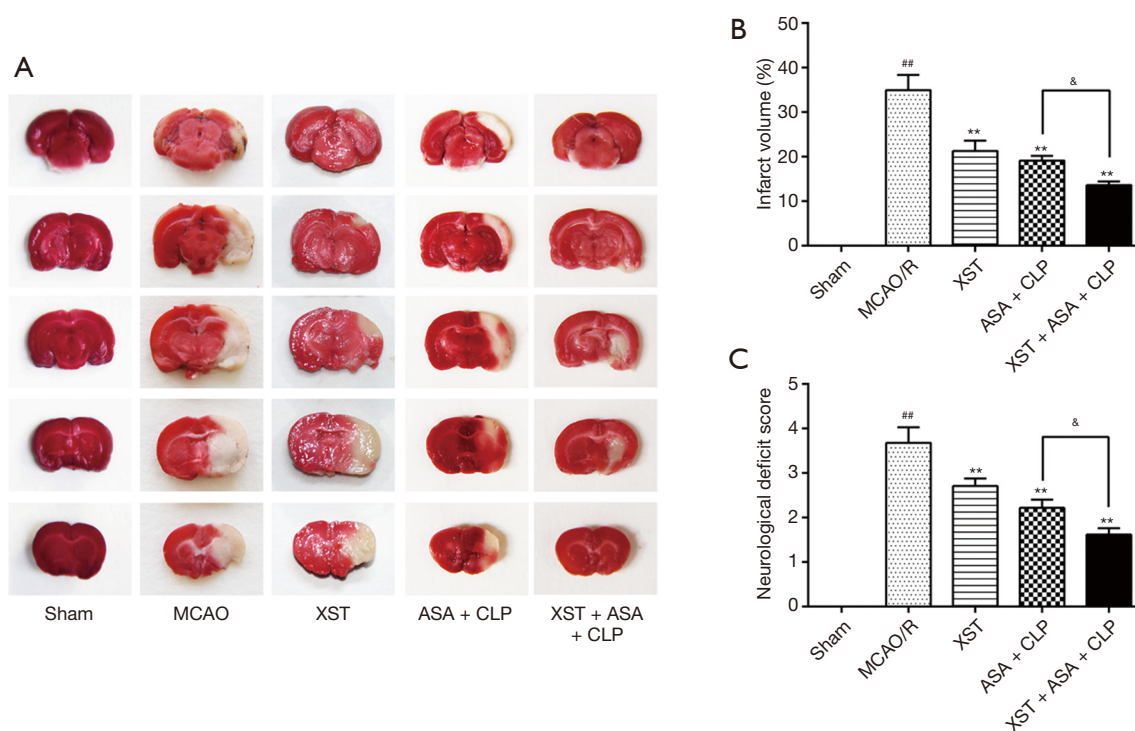


Figure 2 XST+ASA+CLP attenuates MCAO/R-induced infarction volumes and neurologic deficits. (A) Images of TTC staining at 24 h after reperfusion; (B) quantitative analysis of cerebral infarct volumes; (C) neurological score in rats with MCAO/R at 24 h after reperfusion. **, $P < 0.01$, compared with the MCAO/R group; ##, $P < 0.01$, compared with the sham group; &, $P < 0.05$, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; TTC: 2,3,5-triphenyltetrazoliumchloride.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 6.0 (SPAA, Inc., Chicago, Illinois, USA). Data were expressed as means \pm standard deviation (SD) and analyzed by one-way ANOVA followed by Tukey's test. Differences were compared between two groups using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

XST+ASA+CLP attenuates MCAO/R-induced infarction volumes and neurologic deficits

At 24 h after reperfusion, TTC staining and neurologic deficit score were used to measure infarction and neurological function. As shown in Figure 2A,B, the rats that received MCAO treatment revealed a well-demarcated infarct in ischemic core and penumbra ($P < 0.01$). Compared with the MCAO/R group, the XST and ASA+CLP groups exhibited significant decrease in infarction ($P < 0.01$).

Furthermore, the XST+ASA+CLP co-treated group showed significant changes in infarction compared with the ASA+CLP co-treated group ($P < 0.05$). Meanwhile, a visible amelioration in neurologic deficit was observed in XST+ASA+CLP co-treated rats (Figure 2C, $P < 0.05$).

XST+ASA+CLP increases rCBF

As shown in Figure 3A,B, the rCBF in different groups was evaluated using a laser Doppler flowmeter system. The results revealed that the administration of XST, ASA+CLP, and XST+ASA+CLP in treated groups resulted in a significant increase in rCBF at 24 h after reperfusion versus the MCAO/R group ($P < 0.01$). The XST+ASA+CLP co-treated group exhibited more significant increase in rCBF than the ASA+CLP co-treated group.

XST+ASA+CLP attenuates neuronal injury

As shown in Figure 4A, H&E-stained slides of brain sections

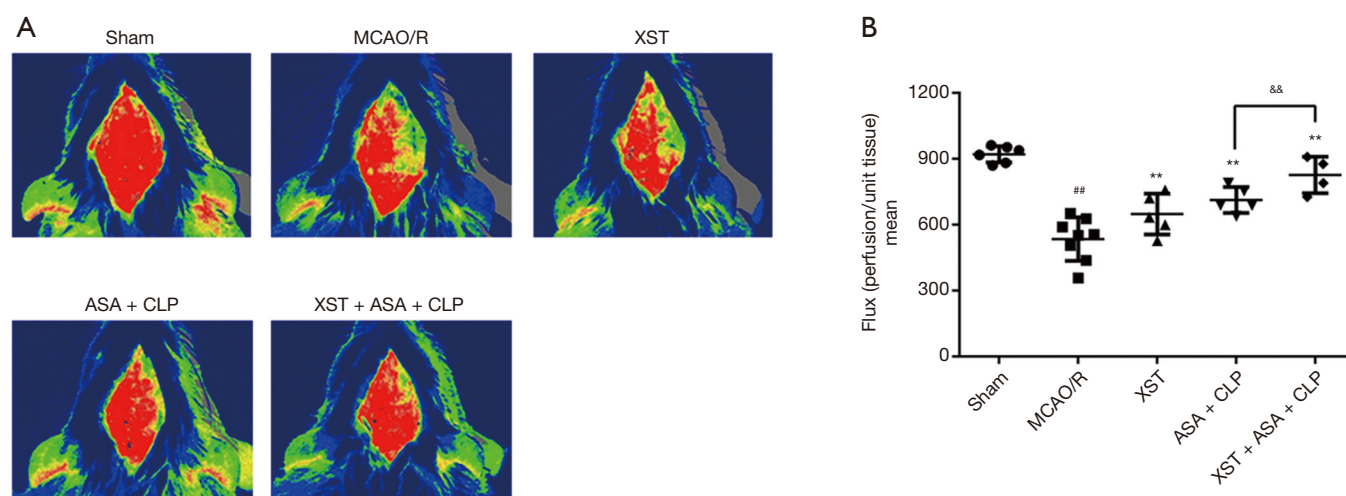


Figure 3 XST+ASA+CLP increases regional cerebral blood flow (rCBF) in rats with MCAO/R. (A) The images of rCBF of cortex. The magnitude of rCBF is represented by different colors, with blue to red indicating low to high. (B) Quantitative analysis of rCBF in different groups. $n=7$ in sham group; $n=5$ in other groups. **, $P<0.01$, compared with the MCAO/R group; ##, $P<0.01$, compared with the sham group; &&, $P<0.01$, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion.

from each group were detected under a light microscope. Untreated rats have many neurons present in the pyknotic nuclei, whereas MCAO/R rats have pale nuclei in the hippocampal CA1 regions. Apparently, administration of XST and ASA+CLP notably reduced the pyknotic nuclei in the hippocampal CA1 regions. Apoptotic damage has been implicated in neuronal dysfunction during ischemic stroke. The apoptotic index of brain tissues was assessed to determine whether the observed better neuronal protection of XST+ASA+CLP against I/R-induced neuronal dysfunction was associated with apoptosis (Figure 4B,C). The number of apoptotic neurons was reduced in drug-treated groups compared with MCAO/R rats. Additionally, the neurological performance of XST+ASA+CLP co-treatment rats also dramatically improved compared with ASA+CLP co-treated rats.

XST+ASA+CLP attenuates activation of microglia and astrocytes

Microglia and astrocytes have been implicated in neuronal death during ischemic stroke (28). The number of microglia and astrocyte cells positively expressed by Iba-1 and GFAP were assessed to determine whether XST+ASA+CLP has better neuronal protection against I/R-induced neuronal dysfunction than the other treatment regimens

(Figure 5). A larger number of Iba-1-positive cells, namely microglia cells, were observed in the cerebral cortex from MCAO/R rats than in those from drug-treated mice ($P<0.01$). The number of astrocyte cells that positively expressed GFAP were distinctly increased in the ipsilateral hippocampus of MCAO/R rats ($P<0.05$). Importantly, attenuated numbers of Iba-1 and GFAP immunoreactivity were discovered in the ipsilateral hippocampus of drug-treated groups ($P<0.05$). The XST+ASA+CLP co-treatment had significantly less quantity of Iba-1 than the ASA+CLP co-treated group ($P<0.05$).

XST+ASA+CLP increases neurotrophic factors expression

Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), and vascular endothelial growth factor (VEGF), or insulin-like growth factor 1 (IGF-1), can irritate multiple growth programs and initiate multiple repair processes, including angiogenesis, neurogenesis, and axonal sprouting (29). In the present study, the expression of various growth factors significantly increased in the peri-infarct region after XST+ASA+CLP treatment compared with ASA+CLP treatment (Figure 6). Hence, XST+ASA+CLP stimulated the further production of neurotrophic factors and enhanced neuronal growth.

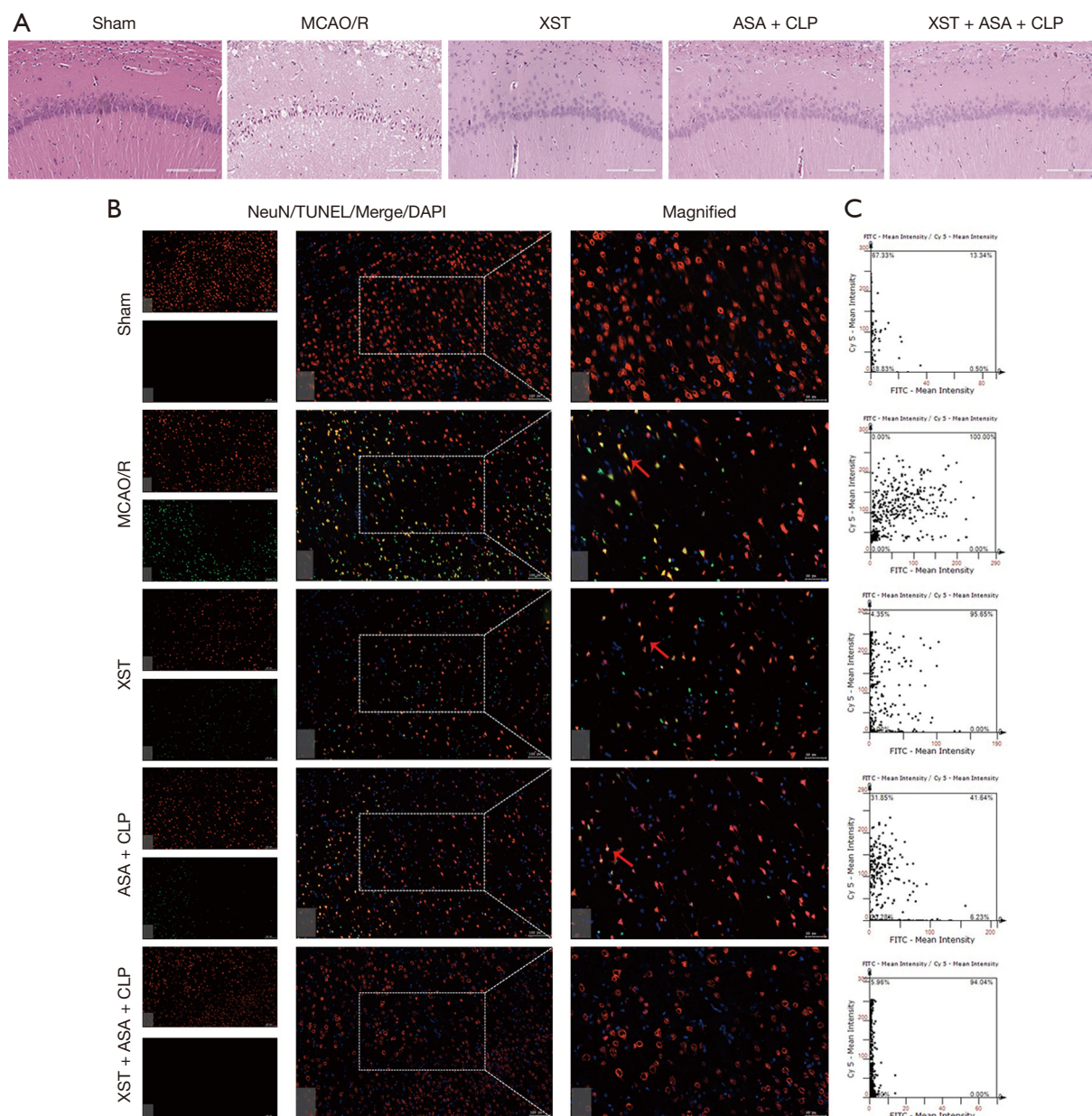


Figure 4 XST+ASA+CLP reduces neuronal injury in the hippocampus and cortex area of rats with MCAO/R. (A) H&E staining in the hippocampal CA1 region in different groups were examined under the light microscope; (B) immunofluorescence for TUNEL-positive neurons in the cortex; (C) quantification of the TUNEL-positive neuron number for different groups. Scale bar: 50 μ m. Values are represented as mean \pm SD for 3 mice in each group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion.

XST+ASA+CLP alters synaptic function and plasticity

A comprehensive understanding of the mechanisms of neurotransmitter transmission and synaptic plasticity led us to assess the development of cerebral ischemic

symptoms and find potential drug targets for improving them. One of the earliest dysfunctional sites in ischemic stroke is the cortical striatal synapse, in which synapses experience multiple forms of plasticity impairment and neurotransmitter transmission disorders (30). These

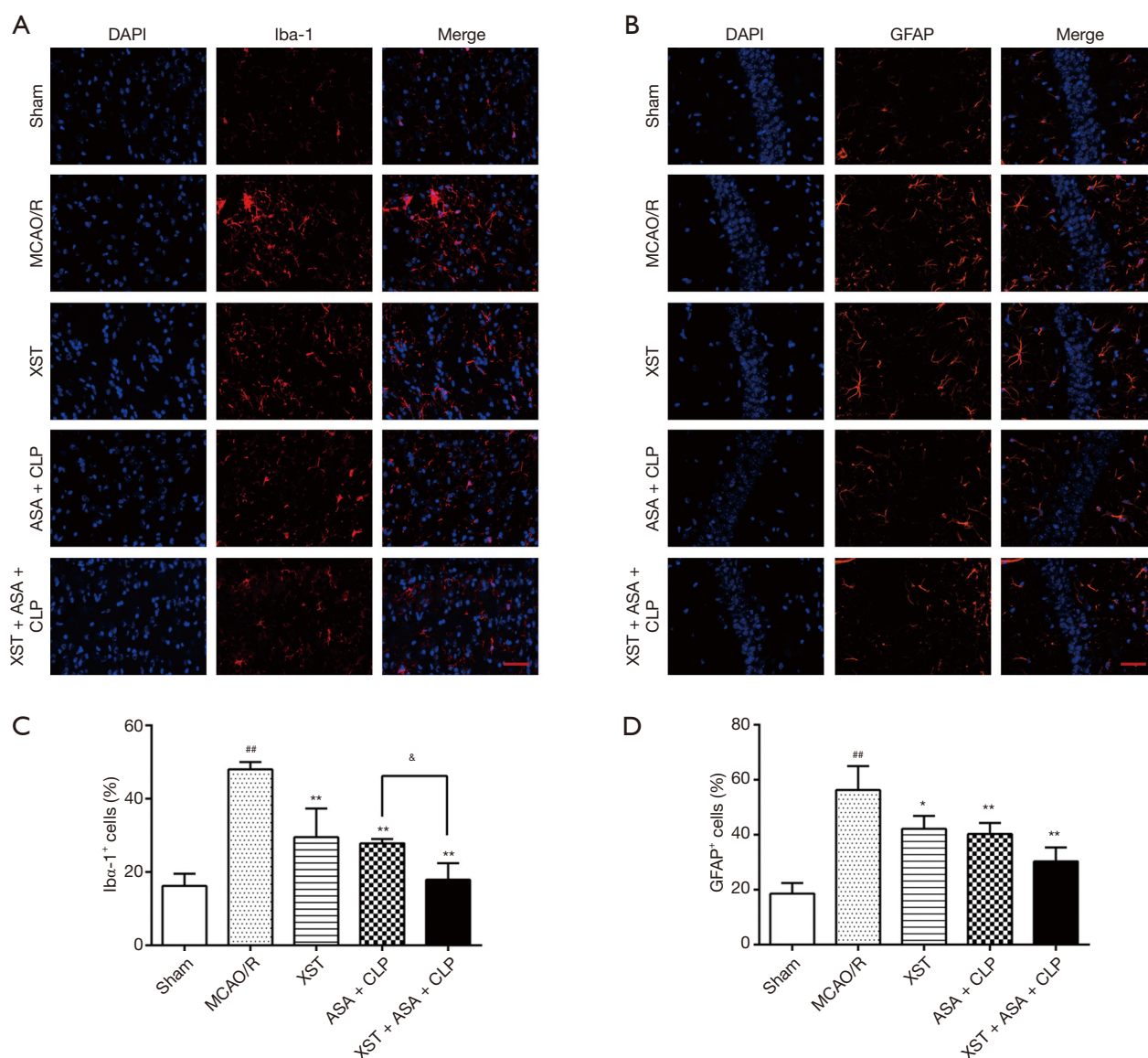


Figure 5 XST+ASA+CLP inhibits the activation of microglia and astrocyte in the cortex or hippocampus area of rats with MCAO/R. (A,B) Immunofluorescence for Iba-1 in the cortex and GFAP in the hippocampus. Scale bar: 50 μ m. (C,D) Statistical analysis of total number of Iba-1 positive cells in the cortex and GFAP positive cells in the hippocampus. Values are represented as means \pm SD for 3 mice in each group. *, $P < 0.05$; **, $P < 0.01$, compared with the MCAO/R group; ^{##}, $P < 0.01$, compared with the sham group; [&] $P < 0.05$, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion.

changes are summarized in *Figure 7*. XST+ASA+CLP altered some cellular signaling pathways involved in neurotransmitter transmission and synaptic plasticity, which improved neuronal dysfunction. This result helped in understanding the pathogenesis of ischemic stroke, especially early cognitive and subsequent cell death caused by excitotoxicity or loss of nutritional support.

XST+ASA+CLP inhibits oxidative stress

During oxidative stress, excess superoxide from NOXs and mitochondria tend to cause lipid peroxidation, DNA oxidation, and protein oxidation. Compared with the sham group, NOX activity and mitochondrial superoxide levels were dramatically increased in the serum of the MCAO/R group. The levels of 4-HNE, a marker of

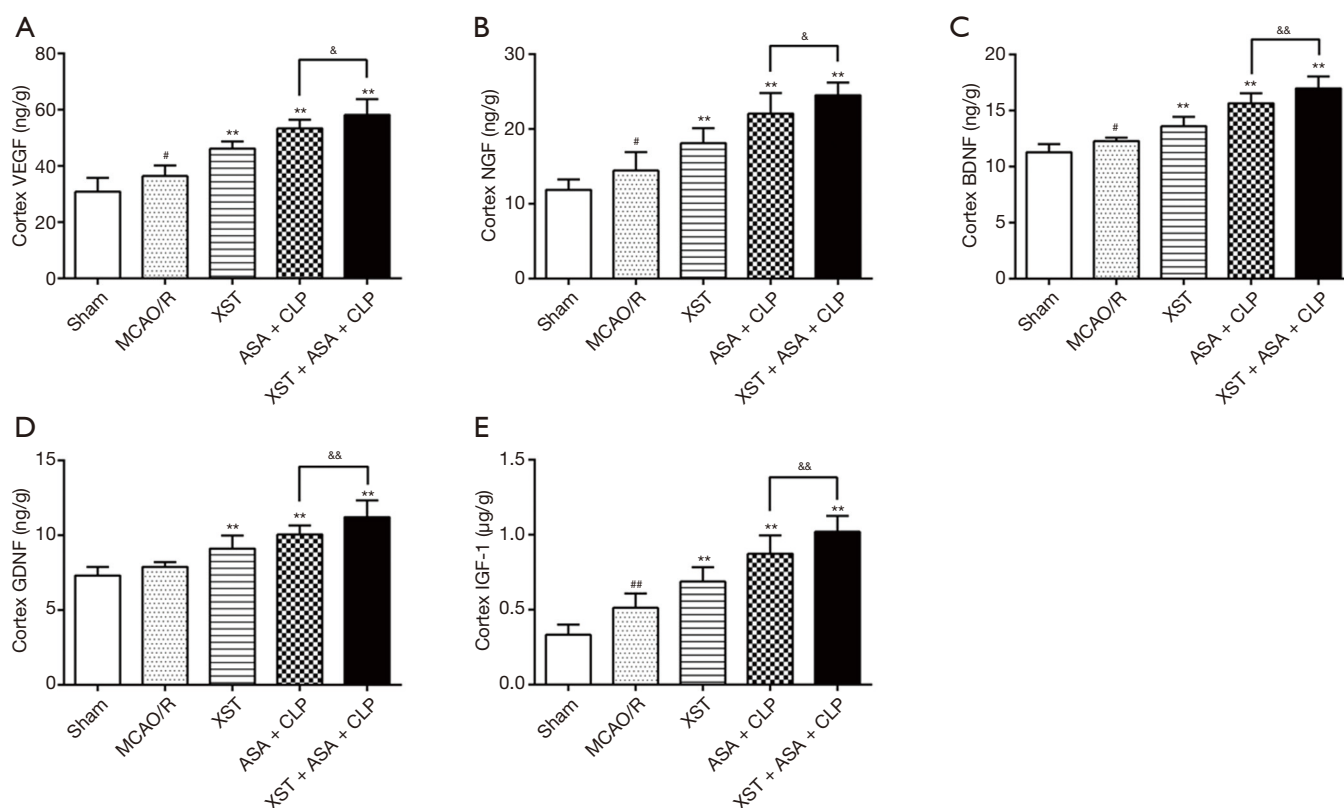


Figure 6 XST+ASA+CLP increases neurotrophic factors expression in rat cortex with MCAO/R. Effect of XST+ASA+CLP treatment on cortex VEGF (A), NGF (B), BDNF (C), GDNF (D) and IGF-1 (E) in rats with MCAO/R. n=6 in sham group; n=8 in other groups. **, P<0.01, compared with the MCAO/R group; #, P<0.05; ##, P<0.01, compared with the sham group; &, P<0.05; &&, P<0.01, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; NGF, nerve growth factor; GDNF, glial cell line-derived neurotrophic factor; VEGF, vascular endothelial growth factor.

lipid peroxidation; protein carbonyl, a marker of protein oxidation; and 8-OHdG, a marker of DNA oxidation products in the serum of rats were remarkably higher in the MCAO/R group (P<0.01). However, 4-HNE, protein carbonyl, and 8-OHdG levels of the drug-treated groups were significantly lower than those of the MCAO/R group (P<0.01). Moreover, NOX and 8-OHdG levels were significantly lower in the XST+ASA+CLP co-treatment group than in the ASA+CLP co-treatment group (Figure 8) (P<0.01).

XST+ASA+CLP inhibits inflammatory factors expression

The protein expression levels of TNF- α , IL-1 β , IL-6, ICAM-1, CD11a, MCP-1, IL-10, and IL-4 in the hippocampus and cortex were not significantly different between MCAO/R-treated and XST+ASA+CLP co-treated mice (Figures 9, Figure S1). However, the expression levels

of IL-6, ICAM-1, CD11a, and MCP-1 in the hippocampus decreased in XST+ASA+CLP co-treated mice compared with those of ASA+CLP co-treated mice (P<0.05). The expression levels of IL-10 and IL-4 in the hippocampus significantly increased in XST+ASA+CLP co-treated mice compared with ASA+CLP co-treated mice (P<0.05). This phenomenon in cortex IL-6, CD11a, MCP-1, and IL-10 was significantly changed in XST+ASA+CLP co-treated mice compared with that in ASA+CLP co-treated mice (P>0.05). Hence, XST+ASA+CLP pretreatment leads to the suppression of brain inflammatory responses during ischemic stroke.

The effects of XST+ASA+CLP co-treated mice on MCAO/R-induced inflammatory response were also detected by the serum levels of circulating inflammatory cytokines (Figure S2). The expression levels of TNF- α , IL-1 β , IL-6, ICAM-1, CD11a, and MCP-1 decreased in rat serum after MCAO/R. By contrast, XST+ASA+CLP

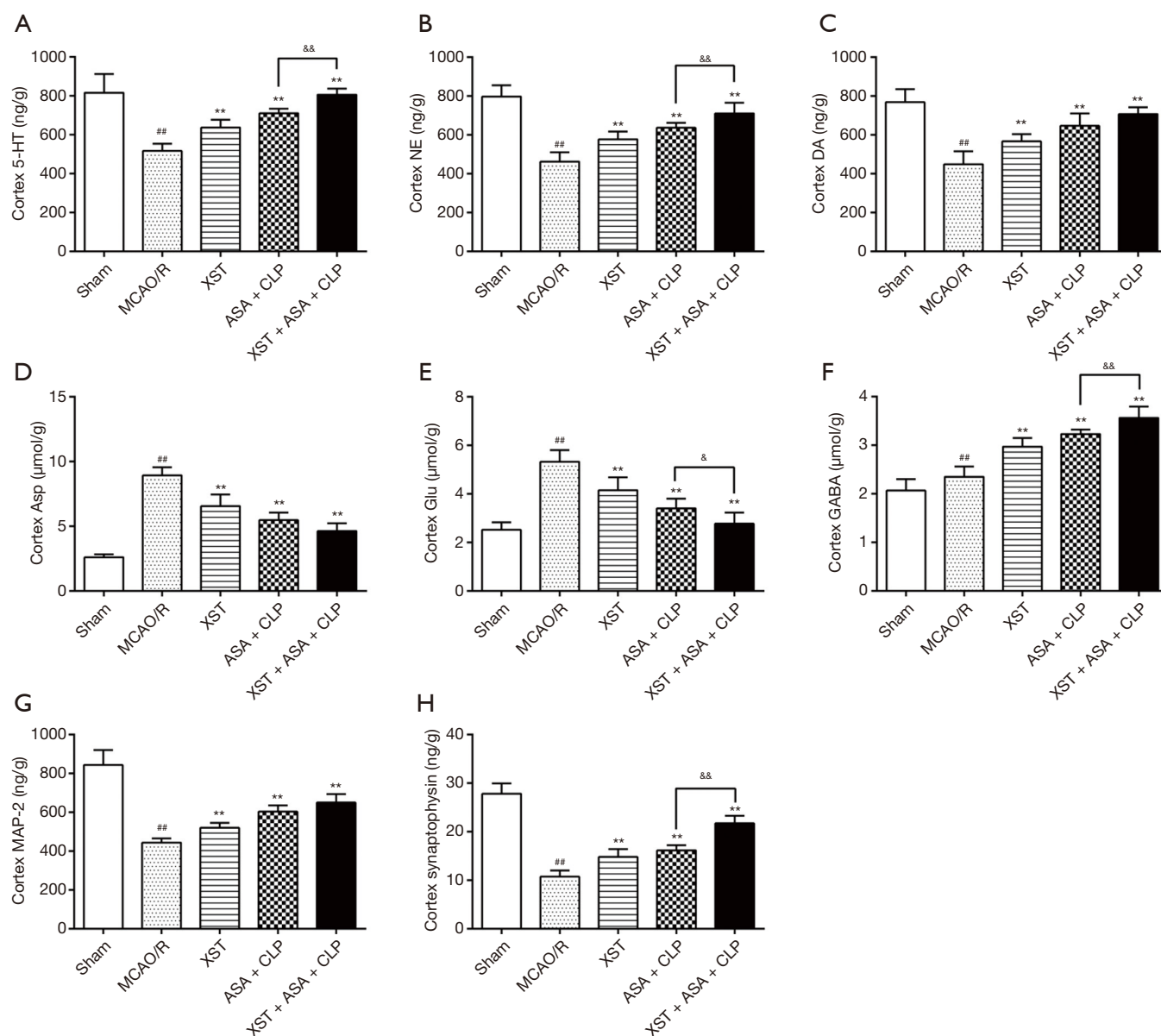


Figure 7 XST+ASA+CLP alters synaptic function and plasticity in rats cortex with MCAO/R. Effect of XST+ASA+CLP treatment on cortex 5-HT (A), NE (B), DA (C), Asp (D), Glu (E), GABA (F), MAP-2 (G) and synaptophysin (H) in rats with MCAO/R. n=6 in sham group; n=8 in other groups. **, P<0.01, compared with the MCAO/R group; ##, P<0.01, compared with the sham group; &, P<0.05; &&, P<0.01, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; 5-HT, 5-hydroxytryptamine; NE, norepinephrine; DA, dopamine; Asp, aspartate; Glu, glutamate; GABA, gamma-aminobutyric acid; MAP-2, microtubule associated protein-2.

pretreatment significantly inhibited the decrease in the serum levels of TNF- α , IL-1 β , IL-6, ICAM-1, CD11a, and MCP-1 caused by MCAO/R. We also measured a significant stimulatory effect of XST+ASA+CLP on the levels of IL-10 and IL-4 in the serum of MCAO/R-treated rats.

XST+ASA+CLP suppresses NOX2 expression and IL-6/STAT3 pathway

The expression levels of NOX2, iNOS, p-STAT3, STAT3, and IL-6 in MCAO/R-treated rats cortex tissues were

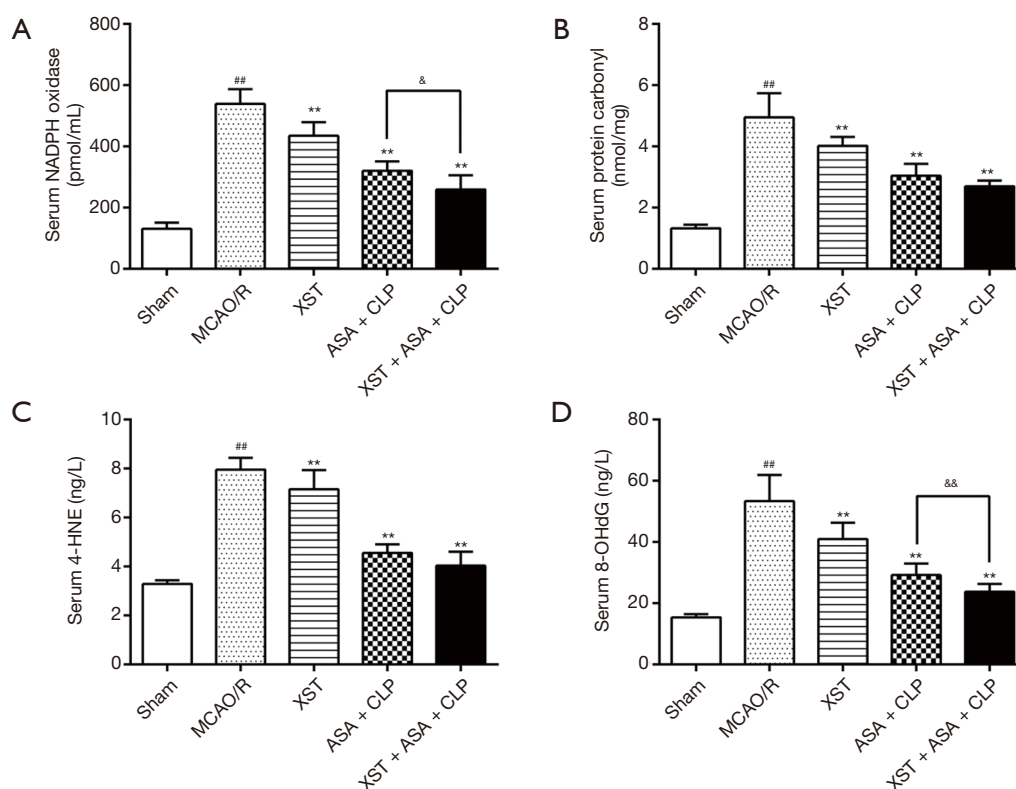


Figure 8 XST+ASA+CLP inhibits oxidative stress in rat serum with MCAO/R. Effect of XST+ASA+CLP treatment on serum NADPH (A), protein carbonyl (B), 4-HNE (C) and 8-OHdG (D) in rats with MCAO/R. $n=6$ in sham group; $n=8$ in other groups. **, $P<0.01$, compared with the MCAO/R group; ^{##}, $P<0.01$, compared with the sham group; [&], $P<0.05$; ^{&&}, $P<0.01$, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; NADPH, nicotinamide adenosine dinucleotide phosphate; 4-HNE, 4-hydroxynonenal; 8-OHdG, 8-hydroxydeoxyguanosine.

measured using Western blot analysis to understand the mechanism of XST+ASA+CLP on ischemic stroke protection (Figure 10). The Western blot results revealed that the XST, ASA+CLP, and XST+ASA+CLP treatments significantly reduced the expression levels of NOX2, iNOS, p-STAT3/STAT3, and IL-6 in the ischemic cortex compared with the MCAO/R group ($P<0.01$). XST+ASA+CLP treatment remarkably upregulated the expression of p-STAT3/STAT3 compared with the ASA+CLP treatment ($P<0.01$).

Discussion

In this study, MCAO/R rat model was used to investigate the effects and possible mechanisms of XST+ASA+CLP following acute ischemic stroke through the suppression of the NOX2/IL-6/STAT-3 pathway. Results show that infarct volume, neurological deficit, rCBF, blood-

brain barrier (BBB) permeability (Figure S3), synaptic function and plasticity, neuronal injury, and glial activation were improved in the model by XST, ASA+CLP, and XST+ASA+CLP pretreatment. Additionally, the pretreated rats exhibited decreased NOX, protein carbonyl, 4-HNE, 8-OHdG, and the activity of various inflammatory factors compared with the MCAO/R group. Furthermore, XST+ASA+CLP treatment has more effectively protected MCAO/R rats than the XST and ASA+CLP treatment. Hence, XST+ASA+CLP could be a new clinical ischemic stroke therapy.

DAPT with ASA and CLP was more frequently applied to patients with different risk factors, placing them at rising risk of recurrent stroke, including prior ischemic events, concomitant vascular risk factors, and index events present in antiplatelet therapy (31). Although DAPT with ASA and CLP co-therapy in early recurrent stroke and coronary artery disease were widely acknowledged, joint use of

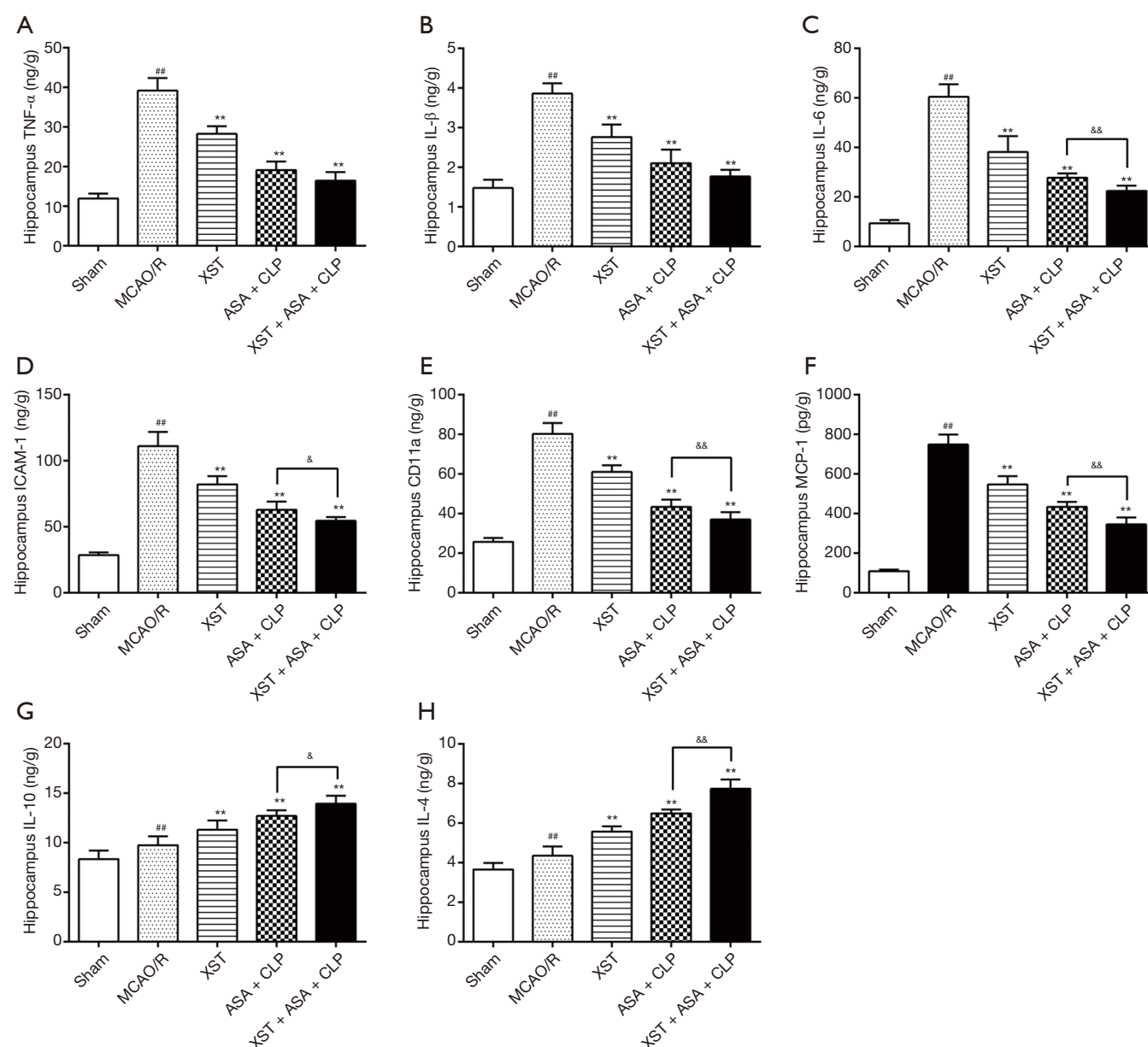


Figure 9 XST+ASA+CLP inhibits inflammatory response in rats hippocampus with MCAO/R. Effect of XST+ASA+CLP treatment on hippocampus TNF- α (A), IL-1 β (B), IL-6 (C), ICAM-1 (D), CD11a (E), MCP-1 (F), IL-10 (G) and IL-4 (H) in rats with MCAO/R. n=6 in sham group; n=8 in other groups. **, P<0.01, compared with the MCAO/R group; ##, P<0.01, compared with the sham group; &, P<0.05; &&, P<0.01, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; TNF- α , tumor necrosis factor-alpha; IL-1 β , interleukin-1 beta; IL-6, interleukin-6; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; IL-10, interleukin-10; IL-4, interleukin-4.

Chinese and Western therapy has been gaining popularity in the recent years. However, the related medicine interactions between DAPT and traditional Chinese medicine were barely known. XST is a freeze-dried saponin

powder injection of *P. notoginseng* saponins, which has been widely used in China for preventing and treating cerebral ischemic stroke (28). XST has a strong neuroprotective function against oxidative stress and inflammation (32,33).

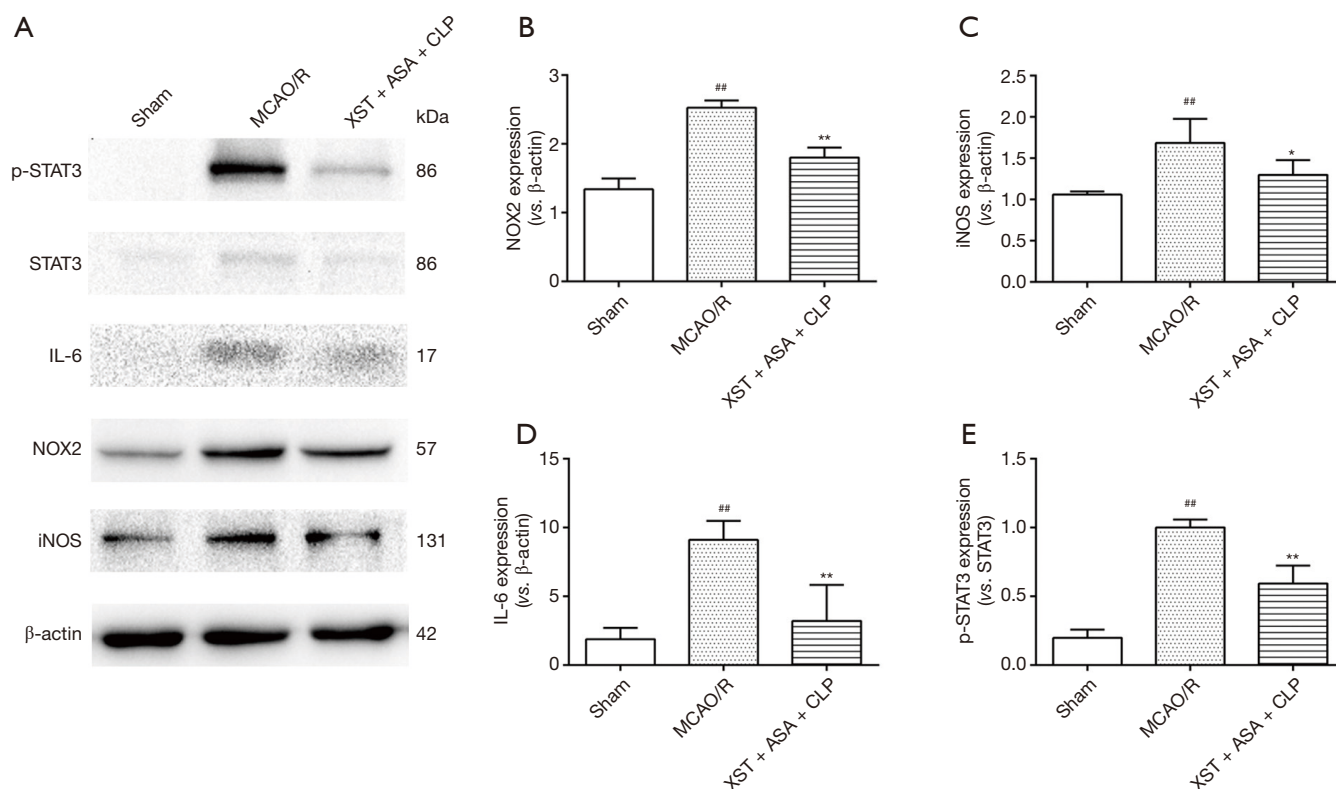


Figure 10 XST+ASA+CLP inhibits the expression of NOX2 and IL-6/STAT-3 signaling after ischemic stroke. (A) Protein expressions of p-STAT3, STAT3, IL-6, NOX2 and iNOS were measured with Western blot; (B) relative protein expression level to β -actin ratios were evaluated. Values are represented as means \pm SD for 3 mice in each group. *, $P < 0.05$; **, $P < 0.01$, compared with the MCAO/R group; ## $P < 0.01$, compared with the Sham group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; NOX2, nicotinamide adenosine dinucleotide phosphate oxidase 2; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase.

Aside from studies that reported the inhibition effect of XST monotherapy on oxidative stress and inflammation, there is no study on the effect of XST and DAPT co-therapy protecting against ischemic stroke against MCAO/R rats by inhibiting oxidative stress and inflammation. Based on the current pharmacological findings, the anti-oxidative and anti-inflammatory effects of XST, ASA+CLP, and XST+ASA+CLP in cerebral MCAO/R injuries were compared, and the relative potential mechanisms of the neuroprotection of XST+ASA+CLP were suggested. More importantly, joint use strategy was investigated to determine whether the drugs could compensate for their individual weaknesses to treat ischemic stroke, or have better outcomes than the monotherapy of drugs, and designed XST and XST+ASA+CLP groups.

Reactive gliosis as a result of ischemic stroke involves astrocytes and microglia, which are pivotal components of

cellular and molecular pathways involved in stroke-induced brain injury (34). The present data confirmed that MCAO/R injury elevated the expression levels of GFAP and Iba-1, whereas the expression of these markers decreased in rats pretreated with XST, ASA+CLP, and XST+ASA+CLP. This phenomenon indicates that the neuroprotective effects of XST, ASA+CLP, and XST+ASA+CLP were attributed to the inhibition of the proliferation of glial cells.

Changes in neuronal activity observed in neurological disorders are associated with many neurotransmitters, including synaptic activity and neuronal plasticity. Neurite outgrowth is a key morphological feature that characterizes neuronal development and an important aspect of neural regeneration that determines neuronal plasticity and neuropathological conditions (35). In the present study, a series of neurochemical mediators and modulators of synaptic transmission were destroyed in

ischemic stroke. Classical neurotransmitters like gamma-aminobutyric acid (GABA), glutamate (Glu), and aspartic acid (Asp); modulators such as 5-hydroxytryptamine (5-HT), dopamine (DA), and norepinephrine (NE); and molecules like BDNF, which indirectly regulates neurotransmission, were destroyed in ischemic stroke. Here, XST with DAPT facilitates the properties of neurotrophic factors. These neurotrophic factors, especially BDNF, promoted synaptic activity and plasticity after stroke and stimulated the proliferation of 5-HT, NE, DA, GABA, microtubule associated protein-2 (MAP-2), and synaptophysin and the downregulation of Asp and Glu. These effects may promote neuronal growth and survival in the developing CNS.

Excessive oxidative stress plays a crucial role in the progression of acute brain injury in both human and animal studies (36). At the molecular level, oxidative stress exacerbates the inflammatory response (37) and triggers the activation of matrix metalloproteinases (38), thereby disrupting the integrity of BBB and accelerating neuronal apoptosis and white matter damage. Basic scientific research on oxidative stress is necessary when studying a prospective intervention in stroke pathology studies (39). Oxidative stress during cerebral MCAO/R injury is characterized by high concentration of ROS, which rapidly overwhelms endogenous antioxidant defense. Intracellular ROS causes oxidation of lipids, proteins, and DNA and alters the final link that promotes neuronal apoptosis signaling pathways (40). Neuronal cells are more likely affected by ROS due to their higher oxidative metabolism and membrane fatty acid content and less antioxidant enzymes (41). Inhibiting the source of ROS is a novel strategy for the treatment of acute ischemic stroke (42). Superoxide is the first ROS produced in the oxygen free radical chain in the early stages of reperfusion. NOX is a direct source of superoxide (7,10) and is activated by a variety of stimuli including angiotensin II, NE, and tumor necrosis factor (43-46), which are related to the progression of neurodegenerative and cardiovascular diseases (47). Increased expression of NOX in rats is related to aggravated ischemic stroke (48). NOX-mutant mice treated with MCAO/R exhibited ameliorative neurobehavioral effect and reduced infarct size (49). NOX isoform NOX2 deletion and superoxide dismutase 2 elevation contributes to neuroprotective effects against memory impairment and hippocampal damage (45). NOX2 is activated and triggers the development of oxidative stress-related damage after subarachnoid hemorrhage (50). The present *in vitro* study has shown that XST, ASA+CLP, and XST+ASA+CLP pretreatments inhibited the increased

levels of oxidative stress markers (such as NADPH, protein carbonyl, 4-HNE, and 8-OHdG) induced by MCAO/R in rats. Moreover, NADPH and 8-OHdG levels significantly decreased in MCAO/R rats co-treated with ASA, CLP, and XST compared with those treated with XST alone and ASA+CLP. These results showed that the neuroprotective effect of XST+ASA+CLP were partly caused by oxidative stress inhibition.

Microglia activation and acute neuronal cell death after MCAO/R injury are related to inflammatory mechanisms (34,51). During this pathological process, inflammatory cascades are triggered by energy depletion and necrotic neuron death in the ischemic region. Therefore, inhibition of pro-inflammatory cytokines, particularly TNF- α , IL-1 β , IL-6, ICAM-1, CD11a, and MCP-1, are likely to have neuroprotective effects during MCAO/R injury. The present study showed that MCAO/R significantly deteriorated neurological function and facilitated microglia activation and neuronal cell apoptosis in the brain. These phenomena were accompanied by the enhanced expression of a series of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, ICAM-1, CD11a, and MCP-1. In contrast, XST, ASA+CLP, and XST+ASA+CLP inhibited all above-mentioned activities induced by MCAO/R challenge. The results also show that the release of anti-inflammatory IL-4 and IL-10 was elevated by XST, ASA+CLP, and XST+ASA+CLP pretreatment. IL-10 attenuates inflammatory milieu in the CNS, inhibits microglia activation, maintains BBB integrity, and lessens neurodegeneration (52). The increase in protein levels of ICAM-1 measured from the serum, cortex, and hippocampus tissues following MCAO/R challenge is consistent with a previous research manifesting that high ICAM-1 levels were more likely to have previous stroke and increased hemorrhagic transformation risk (53). This increase was triggered by MCAO/R challenge and inflammatory cytokine overproduction, which was dramatically limited by XST, ASA+CLP, and XST+ASA+CLP pretreatments. Moreover, inflammatory cytokine levels significantly decreased in MCAO/R rats co-treated with ASA, CLP, and XST compared with those treated with XST alone and ASA+CLP.

To explore the mechanism by which XST+ASA+CLP had better moderating effect on oxidative stress after MCAO/R challenge, we discussed the effects of XST+ASA+CLP on NOX2 expression after MCAO/R challenge. Present results revealed that NOX2 expression was dramatically increased after MCAO/R challenge. Previous results have revealed that NOX2 transforms microglia from a pro-inflammatory

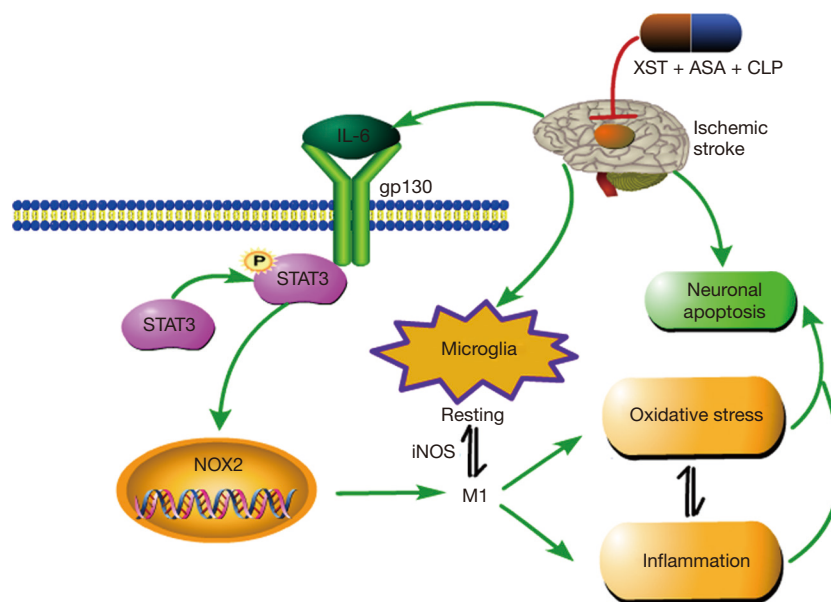


Figure 11 Scheme of mechanisms underlying protective property of ischemic stroke induced by MCAO/R, after XST+ASA+CLP treatment. IL-6/STAT3 pathway increases NOX2 expression in ischemic stage can lead to transform resting microglia to M1 microglia, result in oxidative stress and inflammation. Ultimately, neuronal apoptosis. XST+ASA+CLP suppresses the activation of IL-6/STAT3/NOX2 axis induced by ischemic stroke in rats. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; NOX2, nicotinamide adenosine dinucleotide phosphate oxidase 2; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase.

M1 phenotype to an anti-inflammatory M2 phenotype after traumatic brain injury, and that the weakening of NOX2 inhibits M1 microglia and improves brain damage (54). Interestingly, this previous study reported that microglia, which exhibit pro-inflammatory phenotype, was connected with oxidative stress and pro-inflammatory cytokine expression, leading to the onset of a vicious circle and ultimately to more severe brain damage (Figure 11). In the present study, MCAO/R significantly increased pro-inflammatory microglia and pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 expression in MCAO/R-treated group compared with Sham group. By contrast, XST+ASA+CLP pretreatment remarkably reduced NOX2 expression and reversed inflammatory microglia activation by inhibiting oxidative stress marker and pro-inflammatory cytokines after ischemic stroke.

The IL-6/STAT3 signaling pathway acts as signal transduction in a series of brain injuries, in which STAT3 is a key factor in NOX2 activation (36,55) and can induce pro-inflammatory immune reaction, such as IL-6 (56), which is an important cause of several brain damage activation (57,58). The complex formed by the binding

of IL-6 to the soluble IL-6 receptor is associated with the membrane glycoprotein gp130, which activates JAK phosphorylation coupled to gp130, resulting in STAT3 phosphorylation and leading to the onset and persistence of inflammation (59). NOX2 upstream regulator, the IL-6/STAT3 signaling pathway, was detected to determine how the XST+ASA+CLP treatment inhibits M1 microglia activation after MCAO/R. The present results showed that XST+ASA+CLP pretreatment diminished the expression of phosphorylation STAT3 and IL-6 *in vivo*, as shown by analysis using Western blot. The results suggest that XST+ASA+CLP downregulated NOX2 and that this downregulation is accompanied by the IL-6/STAT3 signaling pathway.

This study also has some limitations. We used an acute rather than a chronic cerebral ischemia model, as the duration of ischemia was 2 hours. Therefore, a long-term cerebral ischemic experiment may have different effects on oxidative stress and inflammation. Furthermore, the signaling pathways associated with oxidative stress and inflammation involve not only NOX2 and IL-6/STAT3 pathways, but also several other mediators that have not

been evaluated in our study, such as Nrf2 and NLRP3. Finally, clinical not just animal experiments should be conducted for a more comprehensive pharmacodynamic evaluation and mechanistic study.

Conclusions

In summary, XST+ASA+CLP showed more neuroprotective activity against oxidative stress and inflammation induced by MCAO/R in rats. These neuroprotective effects were attributed to restrain superoxide formation by inhibiting NOX2 activity. The neuroprotective mechanisms of XST+ASA+CLP involved the inhibition of NOX2 and IL-6/STAT3 signaling pathway after ischemic stroke.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/apm-20-1681>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal care and experimental procedures were reported in accordance with the Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences & Peking Union Medical College and complied with NIH Guidelines for the Care and Use of Laboratory Animals (approval number:

SYXK 2017-0020). All efforts were followed to reduce the number of animals used and ensure minimal suffering.

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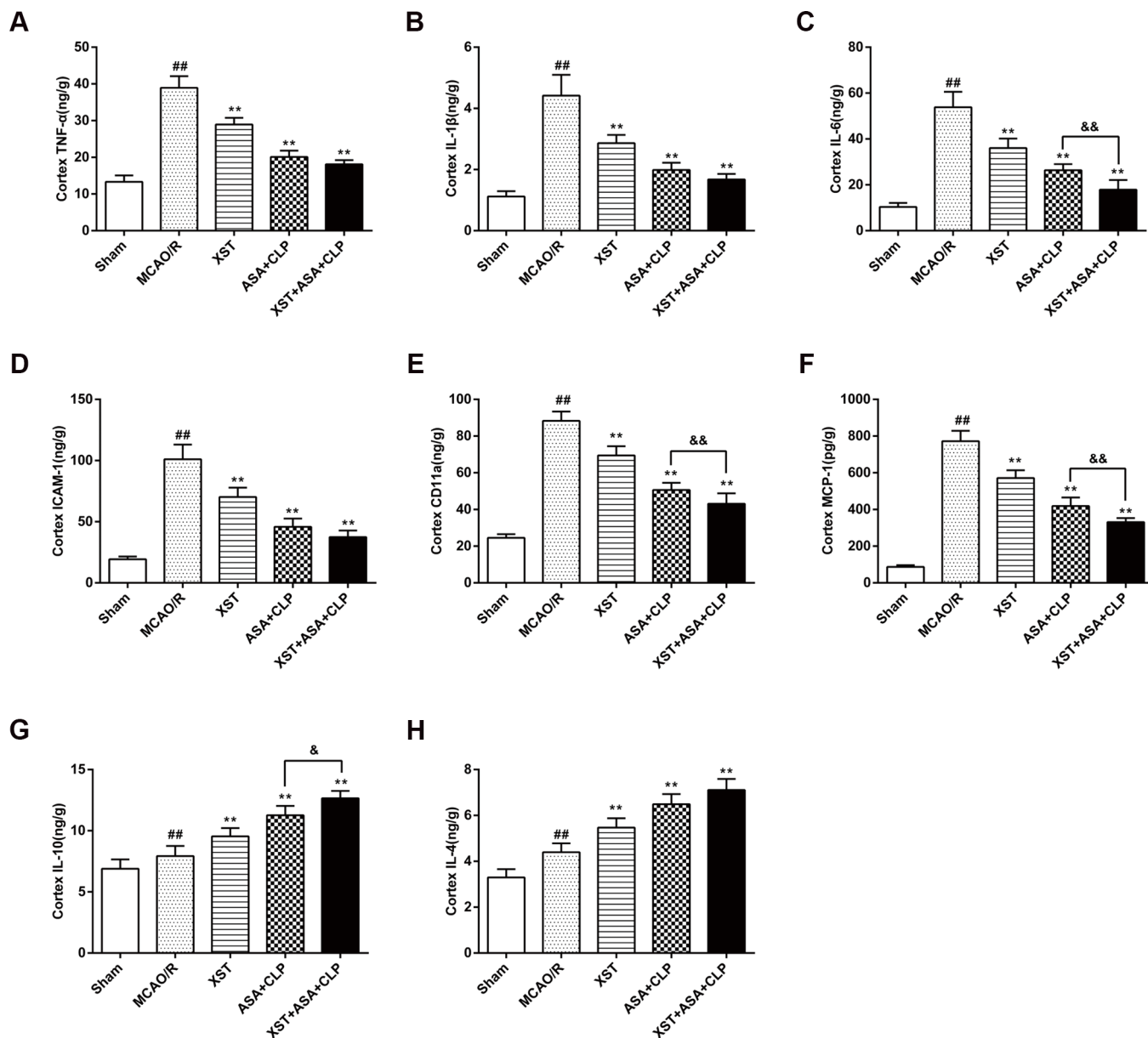


Figure S1 XST+ASA+CLP inhibits inflammatory response in rat cortex with MCAO/R. (A) Effect of XST+ASA+CLP treatment on cortex TNF- α (A), IL-1 β (B), IL-6 (C), ICAM-1 (D), CD11a (E), MCP-1 (F), IL-10 (G) and IL-4 (H) in rats with MCAO/R. Values are represented as means \pm SD for 6 mice in each group. **, $P < 0.01$, compared with the MCAO/R group; ##, $P < 0.01$, compared with the sham group; &, $P < 0.05$; &&, $P < 0.01$, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; TNF- α , tumor necrosis factor-alpha; IL-1 β , interleukin-1 beta; IL-6, interleukin-6; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; IL-10, interleukin-10; IL-4, interleukin-4.

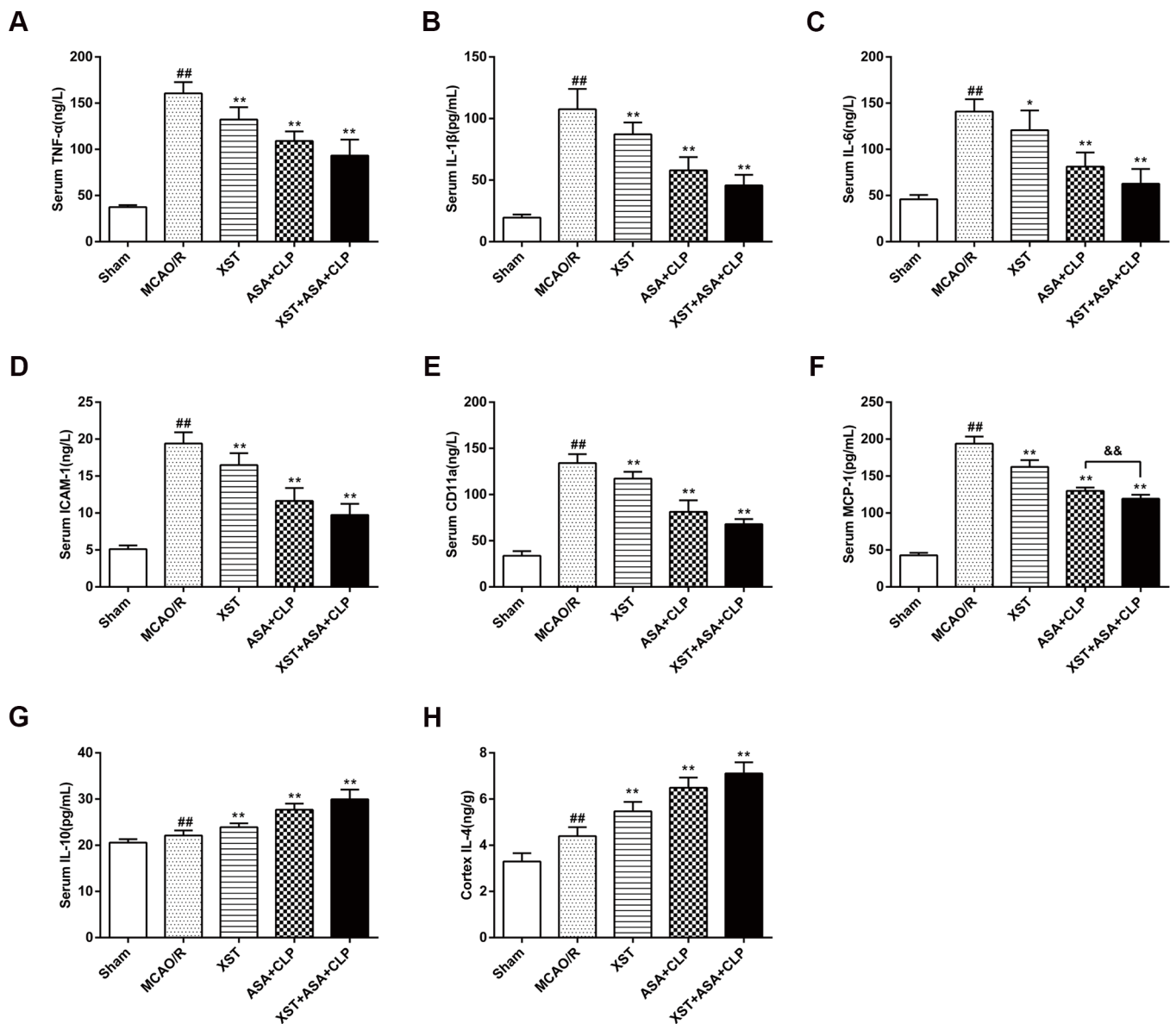


Figure S2 XST+ASA+CLP inhibits inflammatory response in rat serum with MCAO/R. (A) Effect of XST+ASA+CLP treatment on serum TNF- α (A), IL-1 β (B), IL-6 (C), ICAM-1 (D), CD11a (E), MCP-1 (F), IL-10 (G) and IL-4 (H) in rats with MCAO/R. Values are represented as means \pm SD for 6 mice in each group. *, $P < 0.05$; **, $P < 0.01$, compared with the MCAO/R group; ##, $P < 0.01$, compared with the sham group; &&, $P < 0.01$, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; TNF- α , tumor necrosis factor-alpha; IL-1 β , interleukin-1 beta; IL-6, interleukin-6; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; IL-10, interleukin-10; IL-4, interleukin-4.

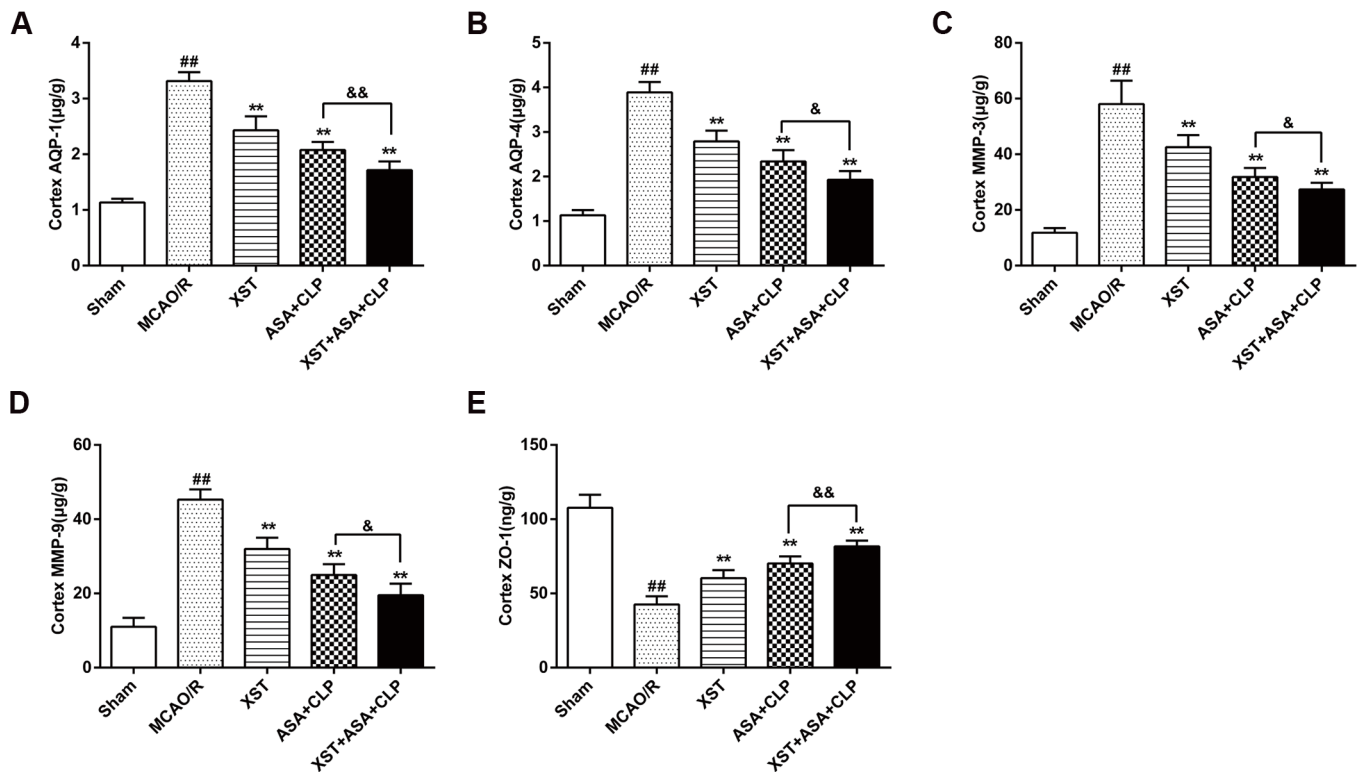


Figure S3 XST+ASA+CLP improves blood-brain barrier permeability in rat cortex with MCAO/R. (A) Effect of XST+ASA+CLP treatment on cortex AQP-1 (A), AQP-4 (B), MMP-3 (C), MMP-9 (D) and ZO-1 (E) in rats with MCAO/R. n=6 in sham group; n=8 in other groups. **, P<0.01, compared with the MCAO/R group; ^{##}, P<0.01, compared with the sham group; [&], P<0.05, ^{&&}, P<0.01, compared with the ASA+CLP group. XST, Xuesaitong injection (lyophilized); ASA, aspirin; CLP, clopidogrel; MCAO/R, middle cerebral artery occlusion/reperfusion; AQP-1, aquaporin-1; AQP-4, aquaporin-4; MMP-3, matrix metalloproteinase 3; MMP-9, matrix metalloproteinase 9; ZO-1, zona occludens 1.