The neuroprotective effect of YaoYi-moxibustion on ischemic stroke by attenuating NK-κB expression in rats

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Background: Traditional Chinese medicine (TCM) has become a crucial direction for ischemic stroke treatment. This study sought to explore the underlying roles of YaoYi-moxibustion (YY-moxi) in ischemic stroke.

Methods: A total of 75 Sprague-Dawley rats were randomly divided into the following 5 groups: (I) the sham-operated group; (II) the middle cerebral artery occlusion model (MCAO) group; (III) the YY-moxi group; (IV) the antioxidant (N-acetylcysteine, NAC) group; and (V) the NAC + YY-moxi group. After the model had been established, the NAC group received intracerebroventricular injections of NAC, the YY-moxi group received YY-moxi, and the NAC + YY-moxi group received a combination of these 2 interventions. The neurological deficit score was confirmed, and the cerebral infarction was examined by triphenyl tetrazolium chloride (TTC) staining. In the ischemia site of stroke, terminal deoxynucleotidyl transferase-mediated Dutp nick end labeling staining was applied to examine the apoptotic cells. Additionally, the apoptosis-associated genes and protein expressions in the ischemic brains were investigated by the reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), immunohistochemistry, and western blot analysis.

Results: YY-moxi alone and YY-moxi combined with NAC significantly reduced the neurological scores and cerebral infarction area of the MCAO rats. Additionally, YY-moxi alone and the combined application of YY-moxi and NAC improved the pathological status of ischemic brain tissues. Further, we found that YY-moxi alone and YY-moxi in combination with NAC could enhanced the antioxidation ability and reduced the inflammatory response of the MCAO model rats. We also proved that YY-moxi alone and YY-moxi combined with NAC significantly suppressed apoptosis-related proteins in the MCAO model rats.

Conclusions: These findings indicate that YY-moxi exerts a protective effect on cerebral ischemic injury by reducing apoptosis. The study suggests that the mechanism may be related to its downregulating the expression of nuclear factor kappa B (NK-κB).

Keywords: YaoYi-moxibustion (YY-moxi); moxa-moxibustion; cerebral ischemic injury; nuclear factor kappa B (NK-κB); apoptosis pathway

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Introduction

Stroke is a common health problem with high morbidity and mortality worldwide (1). According to the World Health Organization, 15 million new strokes occur each year, and 70% of them result in disability or even death (2). Ischemic stroke is the most common type of stroke, and accounts for about 80% of strokes (3). According to research, the pathological mechanisms of ischemic stroke mainly include arterial stenosis occlusion and arterial embolism, which are manifested as the clinicopathological process of local brain tissue hypoxia ischemia and nerve necrosis (4).

At present, stroke unit, intravenous thrombolysis, and interventional therapy have greatly improved the clinical effect of treatments on ischemic stroke (5,6). In addition, Western medicine can restore the blood supply of brain tissue to some extent by anti-platelet aggregation, plaque stabilization, and other treatments (7,8). However, the efficacy of these treatments is not entirely satisfactory in patients with severe ischemic stroke. For example, thrombolytic therapy is only applicable to a few patients, and reperfusion after thrombolysis may also aggravate cerebral ischemia reperfusion injury. Western medicine may cause a large number of adverse reactions, such as gastrointestinal stress ulcer bleeding, and liver and kidney function damage (9,10). In *in-vitro* and *in-vivo* experiments, traditional Chinese medicine (TCM) preparations have been proven to supplement the blood flow to the neovascularization of the brain after stroke, promote nerve growth, and improve the neurological function of animal models and patients (11,12). Thus, effective TCM that can be used in the treatment of ischemic stroke urgently needs to be identified.

At present, YaoYi-medicine has become a part of TCM, and its treatment methods mainly include YaoYi-medicine fumigation, PangTong medicated bath, and Shenhuo moxibustion (13,14). The study found that YaoYi-medicine treatment (especially shenhuo moxibustion) can alleviate symptoms, promote recovery, and decrease the disability rate after ischemic stroke (15). Shenhuo moxibustion, for which the medicinal materials come from the genuine medicinal materials used by yao medicine folk, is used to process the medicinal materials into medicine sticks, to stimulate the acupuncture points with appropriate warm heat, and to cure diseases and provide health care through the conduction of meridians (16). The shenhuo moxibustion method can promote and regulate the immune system, blood circulation, nerve, endocrine system, respiration, digestion, reproduction, and other systems (17). Moxibustion stimulation can promote local skin congestion, capillary expansion, blood circulation and lymph circulation, and enhance the metabolic capacity of local skin tissues (18). Additionally, moxibustion stimulation can reduce the excitability of the nervous system and have sedative and analgesic effects (19). Notably, moxibustion therapy is also beneficial in improving the spasm state of limbs after stroke, promoting the recovery of movement patterns, and reducing the recurrence rate and embolism of stroke (20). However, the underlying mechanism of YaoYi-moxibustion (YY-moxi) in ischemic stroke remains largely undetermined.

In this study, we investigated the possible mechanisms and therapeutic effects of YY-moxi in MCAO model rats. NAC was used as the nuclear factor kappa B (NK-κB) inhibitor. We provide evidence that YY-moxi could significantly relieve ischemic stroke by attenuating NK-κB expression, and thus could be used as a novel therapeutic strategy in the treatment of ischemic stroke. The following article is presented in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3198/rc).

Methods

Animals and grouping

Healthy Sprague-Dawley (SD) rats (n=75), weighing 220 to 250 g, were obtained from the Experimental Animal Center of Guangxi University of Chinese Medicine (Certificate number: SCXK Gui 2009-0002; License: SYKG Gui 2009-2005). The animal experiments were approved by the Animal Ethics Committee of Guangxi University of Chinese Medicine, and were conducted in accordance with the relevant guidelines for the management and protection of experimental animals by Guangxi University of Chinese Medicine. The SD rats were divided into the following 5 groups: (I) the sham group (n=15); (II) the MCAO group (n=15); (III) the MCAO + YY-moxi group (n=15); (IV) the MCAO + NAC group (n=15); and (V) the MCAO + NAC + YY-moxi group (n=15). The animals were allowed to adapt to the laboratory environment for 1 week before the experiment.

MCAO model

As described in a previous study (20), after fasting for 24 h before surgery, the rats were anesthetized by intraperitoneal injection with 2% pentobarbital (45 mg/kg) and fixed in the

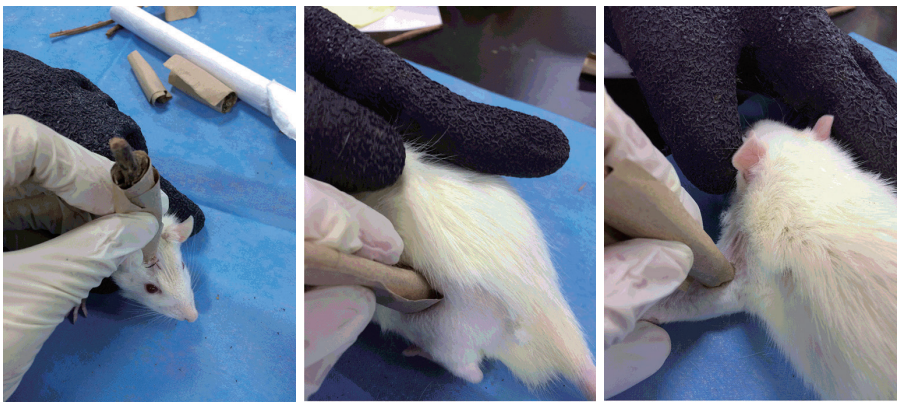

Figure 1 Acupoint selection.

supine position. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were separated and exposed through the midline incision of the neck, and double lines were placed below them for standby. Next, the ECA and the proximal end of the CCA were ligated, and the proximal bifurcation of the CCA was closed by an arterial clip. The nylon threads were treated with 0.1% polylysine and heparin saline and used to block the source of blood supply to the middle cerebral artery. After ischemia for 2 h, the plug was removed for reperfusion. In the sham group, the right CCA and bifurcation were exposed, and the middle cerebral artery was blocked through the insertion wire. The model rats were assessed based on their neurologic deficit scores after 24 h of reperfusion.

Neurological deficits scores

The neurologic deficit scores were evaluated using ZeaLonga (21), and the scores indicated the following: 0 points, no neurological impairment; 1 point, a slight nerve function defect, and an inability to fully extend the left forepaw; 2 points, moderate focal neurological impairment, and circling to left; 3 points, severe focal nerve dysfunction, and dumping to the left; and 4 points, an inability to walk spontaneously, and a decreased level of consciousness. The rats with scores of 1–3 points were indicated successful model establishment, and the corresponding rats were included in the study. Rats with 0, 4 points, or those who died were removed and randomly supplemented.

The production of YY-Shenhuo moxibustion medicine

The YY-shenhuo moxibustion medicine was prepared by the Pharmacy Department of the Third Affiliated Hospital (Liuzhou, Guangxi, China). The preparation method was applied in accordance with the related invention patent (Patent number: ZL2013.1.0729550.2).

Moxibustion treatment and interventions

The "Baihui", "Quchi", and "Zusanli" points of the affected limbs were used as the acupoints (22) (see *Figure 1*). In the YY-moxi group, moxibustion was carried out with YY-Shenhuo moxibustion medicine once a day for 15 min each time for 7 consecutive days. Animals in the MCAO + NAC group and MCAO + NAC + YY-moxi group were given NAC (50 μmol/L) (Sigma, R7150) via intracerebroventricular injection once a day for 3 days. The rats in the control group were injected with normal saline.

Infarct volume determination

The rats were decapitated to remove the brain after the neurological evaluation. Each brain was cut into 2 mm by a special mold, and then each slice was stained by 2,3,5,-triphenyl tetrazolium chloride (TTC) (Sangon Biotech; A610558) for 30 min at 37 ℃ in the dark. The stained sections were arranged in the same background and the infarct volumes were calculated with microscope image-analysis software (Image-Pro plus, USA). The degree of cerebral infarction was expressed as a percentage of the infarct brain volume to the total volume.

TUNEL staining

The paraffin sections were treated with a terminal

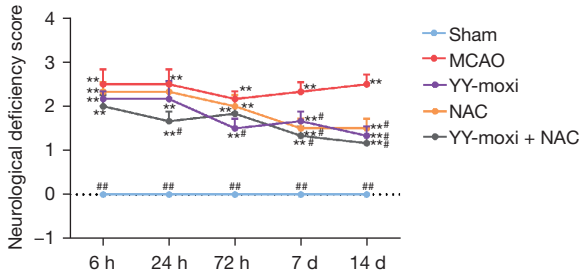

Figure 2 The neurological deficit score of each group. The higher the score, the more severe the dysfunction. **, P<0.01 compared to the sham group; #, P<0.05, ##, P<0.01 compared to the MCAO group. MCAO, middle cerebral artery occlusion model; NAC, N-acetylcysteine; YY-moxi, YaoYi-moxibustion.

deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay kit (Roche; 11684817910) in accordance with the instructions provided by the supplier. The results were visualized using a microscope (Nikon, Japan).

IHC analysis for ischemic brain tissues

Immunohistochemical (IHC) staining was carried out using the IHC MaxVision (TM) method. The fixed tissues were cut into 4 μm sections, and the sections were dewaxed with xylene and treated with 100%, 95%, 90%, 85%, and 75% alcohol for dehydration. The sections were soaked in 1% hydrogen peroxide for 10 min and then placed in citrate buffer for antigen repair. After washing, the sections were treated with normal goat serum at room temperature for 1 h and primary antibodies at 4 ℃ for the night. The next day, after washing, the sections were hatched with secondary antibodies (Abcam, ab6721) for 30 min at room temperature. After staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB), the sections were dehydrated and blocked. The results were examined through an inverted microscope.

Western blot analysis

Total protein was extracted using the protein extraction kit (BestBio; BB-3101). The quantification of protein was analyzed using the Ultra Bradford Protein Assay Kit (Beyotime, P0006). Protein samples (30 μg) in each hole were electrophoresed with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Roche, 3010040001). The blots were sealed with 5% skim milk and incubated with primary antibodies at 4 ℃ for the night and secondary antibodies at room temperature for 1 h. Finally, the expression levels of proteins were detected by electrogenerated chemiluminescence (KALANG; KL-D3490). The primary antibodies included NF-κB/p65 (1:1,000, Abcam, ab16502), Bax (1:1,000, Abcam, ab77566), B-cell lymphoma protein 2 (Bcl-2; 1:1,000, Abcam, ab194583), Caspase-3 (1:1,000, Abcam, ab214430), and glyceraldehyde 3-phosphate dehydrogenase (1:2,000, Abcam, ab9482).

Statistical analysis

All the data in this study are expressed as the mean ± standard error of the mean (SEM). All the results were calculated by GraphPad Prism Software (Ver. Prism 7). The differences between each group were analyzed by a Student's *t*-test or 2-way analysis of variance. A P value <0.05 was considered statistically significant.

Results

YY-moxi protected ischemic cerebral injury in MCAO rats

After the MCAO model was established, the MCAO rats were treated with NAC or YY-moxi for 7 days, and observed until 14 days. Different experiments were performed at 6 h, 24 h, 72 h, 7 days, and 14 days (see *Figure 2*). The neurological deficits of the model group were significantly increased compared to the sham-operated group (P<0.01), but the YY-moxi, NAC, and NAC + YY-moxi groups obviously reduced the neurological deficit scores. TTC staining was performed (see *Figure 3A*). Normal brain tissues appeared red in color, and the infarction region appeared white. In the sham-operated group, each piece of brain slice was uniformly red, and several obvious infarcted areas were found in the brains of the model group. Compared to the model group, the volume of cerebral infarction in the NAC + YY-moxi group was significantly reduced (see *Figure 3B*).

YY-moxi reduced the apoptosis of neurons

Ischemic cerebral injury can induce neuronal apoptosis. The TUNEL staining labeled the apoptotic cells directly; the normal cells were stained in blue, and the apoptotic cells were stained in brown. The results of the TUNEL assays indicated that compared to the sham group, the apoptotic

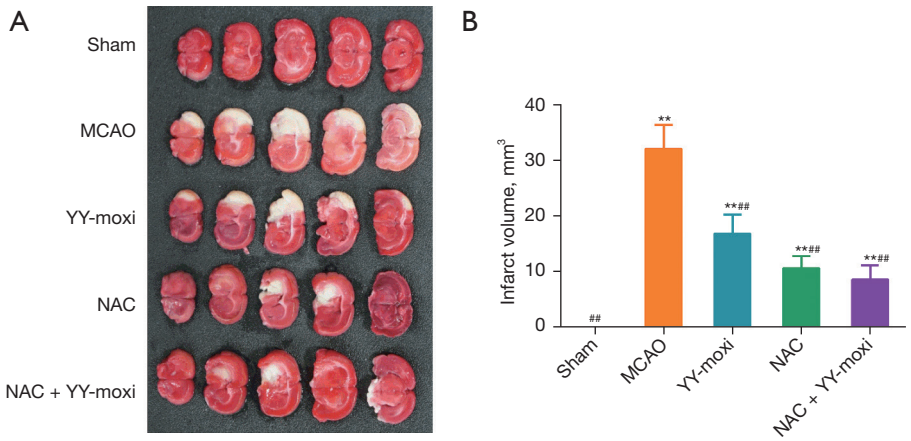

Figure 3 YY-moxi alone and YY-moxi combined with NAC significantly alleviated brain injury in MCAO rats. (A) Rat brain tissue was stained by TTC staining in each group. The pale area is the ischemic infarcted area, and the pink area is normal brain tissue. (B) Cerebral infarction rates of rats in each group. **, P<0.01 compared to the sham group; ##, P<0.01 compared to the MCAO group. TTC, 2,3,5,-triphenyl tetrazolium chloride; MCAO, middle cerebral artery occlusion model; NAC, N-acetylcysteine; YY-moxi, YaoYi-moxibustion.

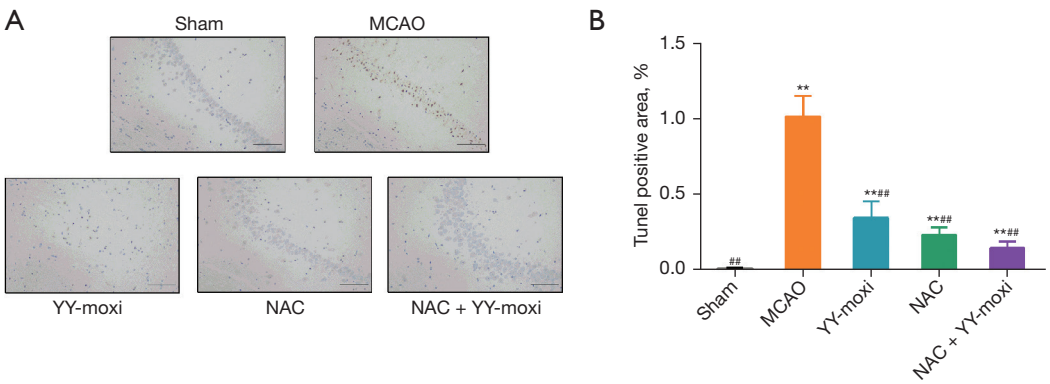

Figure 4 YY-moxi alone and the combined application of YY-moxi and NAC improved the pathological status of ischemic brain tissues. (A) Rat brain tissue was stained by TUNEL staining in each group. Magnification, 40×; scale bar =50 μm. (B) TUNEL assays were carried out to investigate the apoptotic cells in ischemic brain tissues. **, P<0.01 compared to the sham group; ##, P<0.01 compared to the MCAO group. MCAO, middle cerebral artery occlusion model; NAC, N-acetylcysteine; YY-moxi, YaoYi-moxibustion.

cells were prominently increased in the MCAO model rats, but this increase was attenuated by the YY-moxi or NAC treatment, and especially by the combined treatment of YY-moxi and NAC (see *Figure 4*). Thus, the combined treatment of YY-moxi and NAC appeared to significantly reduce the pathological symptoms of ischemic stroke.

Suppressed apoptosis in MCAO model rats related factors

Similarly, we further confirmed the effects of YY-moxi on the expressions of apoptosis-related proteins by conducting IHC assays and western blot (see *Figure 5*). As *Figure 4* shows, the expression levels of Bax and Caspase-3 were significantly upregulated, the expression level of Bcl-2 was observably downregulated in the MCAO group relative to the sham group, while moxa-moxi and YY-moxi treatment weakened the upregulation of Bax and Caspase-3 levels and the downregulation of Bcl-2 level in the MCAO model rats. The results showed that Bax and Caspase-3 were downregulated, and Bcl-2 was upregulated in the YY-moxi

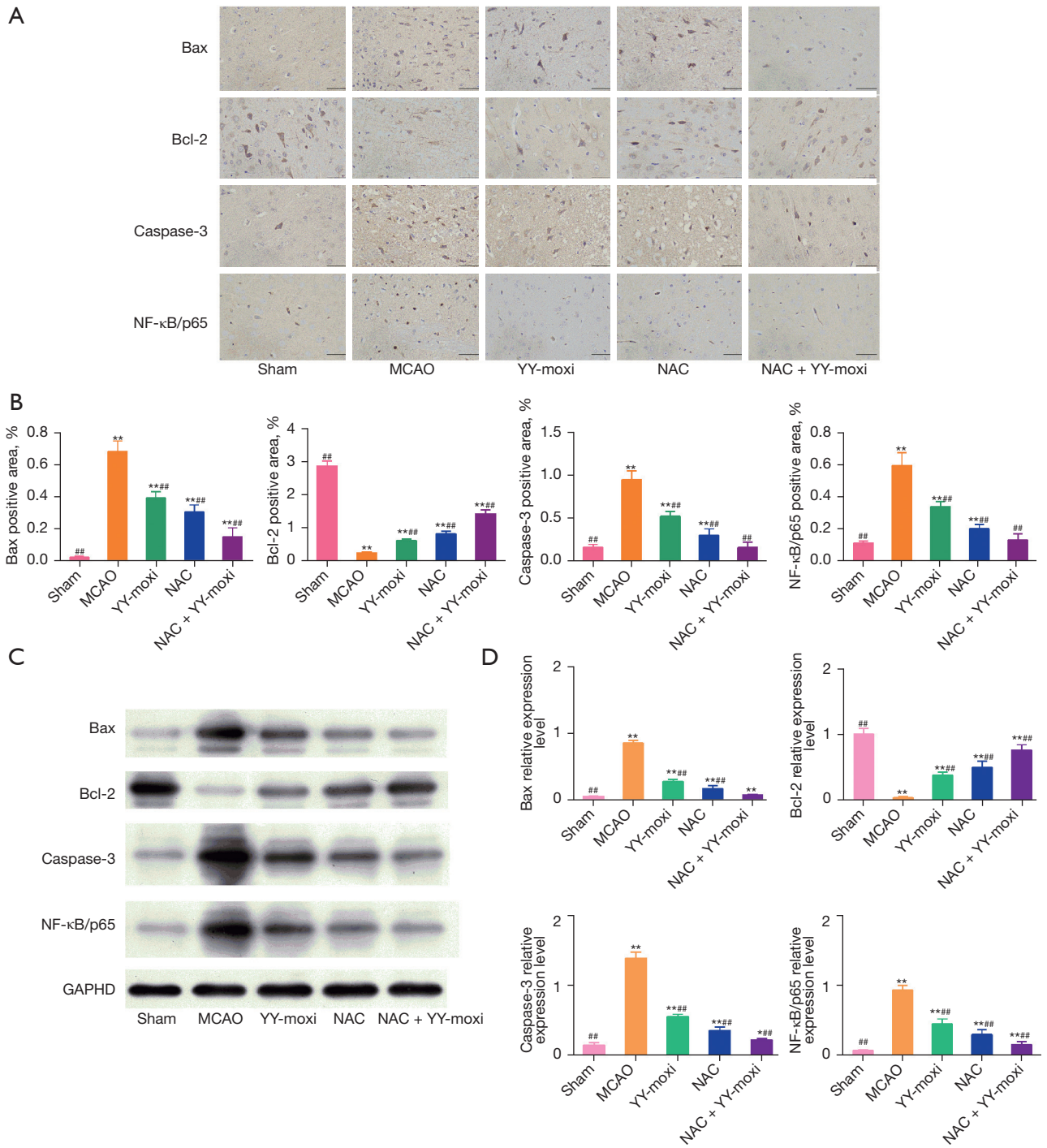

Figure 5 YY-moxi alone and YY-moxi combined with NAC significantly downregulated NF-κB/p65 levels, increased Bcl-2, and decreased Bax and Caspase-3 levels in MCAO model rats. (A) The levels of Bax, Bcl-2, Caspase-3, and p56 were assessed by IHC assays in the brain tissue of each group. Magnification, 40×; scale bar =50 μm. (B) The positive areas of Bax, Bcl-2, Caspase-3, and p56 were quantitatively analyzed according to the IHC results. (C) Protein expression of Bax, Bcl-2, Caspase-3, and p56 in the injured brain tissue of each group. (D) Western blot analyses of the apoptosis-associated in the MCAO model rats. *, P<0.05, **, P<0.01 *vs.* the MCAO group; $^{\#\#}$, P<0.01 *vs.* the YY-moxi group. IHC, immunohistochemical; MCAO, middle cerebral artery occlusion model; NAC, N-acetylcysteine; YY-moxi, YaoYi-moxibustion.

group. Thus, we proved that YY-moxi markedly inhibited the apoptosis of the MCAO model rats.

Discussion

Based on the previous studies (13,14) and the preliminary results of our experiment, YaoYi Shenhuo moxibustion inhibited neuronal apoptosis, reduced the cerebral infarct area, improved neurological functional score, upregulated the expression of Bcl-2, and decreased the level of Bcl-2, Bax and caspase-3. Similarly, the NF-κB antagonist NAC strengthened the improvements of the neurological functional score, inhibited neuronal apoptosis, reduced the cerebral infarct area and the changes in NR2B levels caused by ischemic cerebral injury. Thus, YY-moxi might exert a neuroprotective effect in cerebral ischemic injury by inhibiting apoptosis in a rat model of stroke by regulating the target of NF-κB.

Cerebrovascular disease is 1 of the 3 major diseases threatening human health, and has high morbidity, high disability, and high mortality (23). Ischemic stroke accounts for 2/3 of all cerebrovascular diseases (24). At present, there is still no definite treatment for ischemic stroke, so it is necessary to search for novel brain protection treatments (25). NAC, a precursor of glutathione, is an antioxidant that can reduce oxidative stress and inflammation (26). A study has certified that NAC administration before ischemia can reduce cerebral injury caused by ischemia and reperfusion (27). However, the antioxidant has not been shown to have an effective therapeutic effect in clinical trials of stroke. Thus, an effective method of treating ischemic cerebral injury is required to target multiple pathological changes.

YaoYi Shenhuo moxibustion is a folk technique of Yao nationality in Guangxi province, which has the functions of expelling wind and cold pathogens, activating blood circulation to dissipate blood stasis, warming the arteries, clearing the veins, dispelling wind, and relieving pain. At present, it has achieved good therapeutic effects in the treatment of stroke patients with hemiplegia. In our study, we further investigated the underlying neuroprotective effects and molecular mechanisms of YY-moxi alone and in combination with NAC on ischemic cerebral injury. A recent study verified that moxa-moxibustion is an adjunctive therapy in the treatment of stroke, which can significantly improve neurological deficits in stroke patients (28). A clinical study has also been reported that YY-moxi combined with synthetic rehabilitation therapy could improve limb movement function in patients with shoulder-hand syndrome after stroke (29). Our results also revealed that YY-moxi alone and combined with NAC contributed to the neurological and pathological improvements of MCAO rats, and its therapeutic effect on ischemic cerebral injury was stronger than moxibustion. Thus, YY-moxi alone and in combination with NAC was found to have a good therapeutic effect on ischemic cerebral injury.

In the pathogenetic process of ischemic cerebral injury, a hypoxic and ischemic microenvironment leads to changes in the signaling pathways in the infarct and ischemic penumbra neurons, which is a main cause of neuron degeneration and apoptosis (30). The intervention and adjustment of these changes (i.e., via the activation of the intracellular survival signaling pathways or/and the inhibition of the death pathways) are beneficial in protecting brain neurons, relieving inflammation, inhibiting apoptosis, and reducing mortality and disability (31).

NF-κB is a vital transcriptional regulator, which participates in the processes of cellular immune response, growth, differentiation, and apoptosis (32,33). The key modulator of the NF-κB pathway is associated with human cases of ischemic cerebral injury. It has been suggested that in human stroke patients within 48 h following stroke increase NF-κB in the penumbra region. Like people, mice lacking the p50 subunit of NF-κB develop significantly smaller infarcts after transient focal ischemia (34). Clearly, NF-κB signaling plays a vital role in tissue viability and recovery from ischemic insult in humans, and also in the rodent models of ischemic cerebral injury.

Previous research has also shown that spontaneously hypertensive rats, which is a common strain of rats to model hypertension in ischemic cerebral injury studies, have higher levels of matrix metalloproteinase activity due to the increased activation of NF-κB (35). This demonstrates that the inhibition of NF-κB signaling reduces ischemic cerebral injury, reduces the cerebral infarct area, and neuronal death in MCAO rats.

The inhibition of apoptosis by NF-κB signaling is a complex process involving multiple signaling pathways. The inhibitory effect of NF-κB on apoptosis may also be reflected in its transcriptional activation of its downstream apoptotic genes in the Bcl-2 family, which is related to gene products, also have anti-apoptotic effects through the mitochondria and upstream of caspase-3. The mitochondrial apoptosis pathway is a major apoptosis pathway and Bax, Bcl-2, Caspase-3 proteins are 3 important apoptosis-related factors (36,37).

When the cells start apoptosis, the equilibrium state between Bax and the Bcl-2 protein in the mitochondrial membrane is disrupted. As a pro-apoptotic protein, the expression of Bax will substantially increase, and as an anti-apoptotic Bcl-2 protein, its level will predominantly reduce (38-40). Mitochondrial abnormalities activate the downstream pro-apoptotic factor of Caspase-3 (41). The activation of Caspase-3 leads to deoxyribonucleic acid breaks in nuclear and the promotion of cell apoptosis (42). Research has also demonstrated that the inhibition of the NF-κB signaling pathway can reduce the cerebral infarction area and neuronal death in MCAO rats, and reduce ischemia reperfusion injury (43).

In conclusion, this study showed that YY-moxi reduced neurologic deficit scores, neuronal apoptosis, and the cerebral infarction rate induced by cerebral ischemia/reperfusion in rats. YY-moxi downregulated the levels of Bax protein, upregulated the level of Bcl-2 protein, and downregulated the levels of caspase-3, and p65 protein. Similar to the effects of the NF-κB antagonist NAC, it is believed that YY-moxi may be an effective neuroprotective measure for the treatment of ischemic stroke. In our study, we found that YY-moxi alone and combined with NAC markedly enhanced anti-apoptotic ability. The neuroprotective mechanism of YY-moxi is related to its downregulating the expression of NF-κB. The optimal treatment time for YY-moxi therapy for ischemic stroke has not yet been completely elucidated. In addition to the apoptosis pathway and the pathway related to the NF-κB pathway, the use of YY-moxi therapy to treat ischemic stroke damage requires further research.

Conclusions

The protective effects of YY-moxi alone and in combination with NAC on MCAO model rats included reducing neuronal apoptosis by regulating the NF-κB signal pathway. Thus, YY-moxi alone and the combination YY-moxi and NAC might be potential treatments for ischemic stroke.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3198/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3198/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3198/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work, including ensuring that any questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved. The animal experiments were approved by the Animal Ethics Committee of Guangxi University of Chinese Medicine, and were conducted in accordance with the relevant guidelines for the management and protection of experimental animals by Guangxi University of Chinese Medicine.

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