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Neuroprotective effect and mechanism of Mu-Xiang-You-Fang on cerebral ischemia-reperfusion injury in rats

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ABSTRACT

Ethnopharmacological relevance: The present study is to investigate the neuroprotective effect of Mu-Xiang-You-Fang (MXYF), a classic Traditional Chinese Medicine used by Chinese minorities to treat stroke, on cerebral ischemia-reperfusion (I/R) injury and the related signaling pathways.
Materials and methods: Male Sprague-Dawley rats were divided into 6 groups: sham group, I/R group, nimodipine and MXYF (58, 116 and 232 mg/kg respectively) groups. Cerebral ischemia model was induced by middle cerebral artery occlusion for 2 h followed by reperfusion for 48 h. Neurological functional score was evaluated according to the method of Zea longa's score and the infarct area was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining at 48 h after reperfusion. The protein expression of cytochrome c (cyt-c), Bcl-2, Bax, caspase-9, caspase-3 and caspase-7 were analyzed by western blot and the mRNA expression of Caspase-9, Caspase-3 and Caspase-7 were determined by the reverse transcription-polymerase chain reaction.
Results: Oral administration of MXYF (116 and 232 mg/kg) significantly reduced the neurological functional score and attenuated the cerebral infarct area. Western blot analysis showed that the expression of Bcl-2 is enhanced and Bax expression is inhibited after treatment with MXYF (116 and 232 mg/kg), leading to significant increase of the ratio between Bcl-2 and Bax. Furthermore, the protein expression of cyt-c, caspase-9, caspase-3 and caspase-7 was significantly inhibited while the mRNA expression of caspase-9, caspase-3 and caspase-7 but not cyt-c was markedly inhibited in the MXYF (116 and 232 mg/kg) treatment groups compared with the I/R group.
Conclusions: The above data suggested that MXYF has potential neuroprotective activities by the regulation of apoptotic pathway, MXYF is a promising agent in treatment of stroke.

1. Introduction

Stroke, a sudden severe cerebral illness of vascular origin (Nattrass, 1953) is one the most common causes of death worldwide. It can also cause permanent disability in survivors (Hua et al., 2015; Murray and Lopez, 1997), leading to bad life quality of the survivors and tremendous need of further treatment. Among those treatments, cerebral blood flow reperfusion is the first choice for the treatment of ischemic stroke. Unfortunately, reperfusion in the ischemic brain can induce a cascade of pathophysiologic processes resulting in further damage known as ischemia-reperfusion (I/R) injury (Zhang et al., 2014a). Chemical drugs such as neuro-protective agents, radical scavengers or calcium ion antagonist have been used for the treatment of cerebral I/R injury. However, side effects such as cerebral hemorrhage, resistance to drugs and gastrointestinal irritation arise as new problem in the long-term therapy. Furthermore, it is difficult to achieve significant therapeutic results when used single chemical drug alone (Lan et al., 2014).

Apoptosis plays an essential role in the pathogenesis and prognosis of different cerebrovascular diseases including chronic and acute ischemia (Li et al., 2014). Bcl-2 family of proteins (including Bcl-2 and Bax) control cell proliferation, differentiation and programmed cell death during normal brain development (Mishra et al., 2006). Bcl-2 prevents apoptosis by forming a heterodimer with the pro-apoptotic protein Bax and protects cells from programmed cell death induced by hypoxia (Farlie et al., 1995; Martinou et al., 1994), The relative ratio between Bcl-2 and Bax in the cells determines whether the cells will survive or undergo apoptosis (Almawi et al., 2004).

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Apoptosis is initiated by the release of cytochrome c (cyt-c) from the mitochondrial intermembrane space. Cyt-c associates with Apaf-1 in a dATP dependent manner to promote the activation of caspase-9 which cleaves and activates the executioner caspases-3 and caspase-7. Ischemia can upregulate caspase-9 in human brain tissue (Chen et al., 2015), and caspase-3 is a key mediator of apoptosis in animal models of ischemic stroke. Both genetic disruption and pharmacological inhibition of caspases have a strong neuroprotective effect in experimental stroke (Endres et al., 1998; Ma et al., 1998; Sugawara et al., 2002). Modulation of Bcl-2/Bax and suppression of cyt-c, caspase-9, caspase-3 and caspase-7 expressions may have a protective role in the treatment of cerebral I/R injury.

Traditional Hui Medicine (THM), a branch of Traditional Chinese Medicine (TCM), is an integration of the TCM and the Arab medicine which has been used in China for about six hundred years. This kind of Chinese medicine is efficient in the treatment of encephalopathy, especially stroke (Wang et al., 2014). Mu-Xiang-You-Fang (MXYF) is one of the classical THM formulas of THM that has high efficiency in the treatment of stroke (Song, 2000; Zhang et al., 2014b). According to the record of Hui Yao Fang, a Chinese Hui medical classic, MXYF is made of five medicinal herbs, including *Aucklandia lappa* Decne., *Piper nigrum* L., *Euphorbia kansui* T. N. Liou ex T. P. W.

In this study, we tested the neuroprotective activities of MXYF on cerebral ischemia induced by middle cerebral artery occlusion (MCAO), and investigated whether it exerted the neuroprotection by inhibiting apoptosis. In addition, we examined the effects of MXYF on cyt-c, Bcl-2, Bax, caspase-9, caspase-3 and caspase-7 expressions in the ischemic penumbra of brain to elucidate the possible neuroprotective mechanisms.

2. Materials and methods

2.1. Plant material and extraction

Aucklandia lappa Decne (voucher specimen no. 20,140,308), *Piper nigrum* L. (voucher specimen no. 20140213), *Euphorbia pekinensis* Rupr (voucher specimen no. 20140115), *Callorhinus ursins* Linnaeus (voucher specimen no. 20140422), *Asarum heterotropoides* Fr. Var. *mandshuricum* (Maxim.) kitag. (voucher specimen no. 20140302) were purchased from Anhui taiyuan Chinese Herbral Pharmological Co., Ltd. All of them were identified by Prof. Yunsheng Zhao, school of pharmacy, Ningxia Medical University, and were conserved in Ningxia Hui Medicine Modern Engineering Research Center. According to the previous study (Song, 2000), the *A. Lappa*, *P. Nigrum*, *E. Pekinensis*, *C. ursins* and *A. Heterotropoides* were mixed at the ratio of 3:1:1:1:1 (w/w, with a total weight of 46 g) and then smashed the mixture into coarse dust, then the mixture was extracted by supercritical CO_2 fluid extraction apparatus with a pressure of 30 MPa under 50 °C for 2 h, the yield of volatile oil was 2.90%. The daily dose of MXYF is (46 × 2.90%) g/75 kg body weight. According to the formula: $d_{rat} = d_{human} \times 0.71/0.11$, the dose of MXYF for rat should be 116 mg/kg. In the present study we used 58, 116, 232 mg/kg as low, middle and high dosages for rat, respectively. To ensure the quality and stability of the MXYF solution, we used GC-MS to identify the active compounds.

The main components of MXYF extract were identified by GC-MS. The mass spectrometric conditions were set as follows: Shimadzu QP2010 plus GC-MS, Rxi-5Sil MS quartz capillary column (30 m × 0.25 mm × 0.25 μm), the starting temperature is 50 °C, maintain for 1 min, heat to 150 °C with 8 °C min^{-1}, maintain for 3 min, then heat to 210 °C with 3 °C min^{-1}, maintain for 3 min, heat to 280 °C with 15 °C min^{-1}, maintain until the end of analysis; helium as the carrier gas, column flow rate is 1.0 ml min^{-1}, split ration is 25:1, inlet temperature is 250 °C, EI ionization source is 70 eV, ion source temperature is 230 °C, scan range of *m/z* 35–500.

2.2. Reagents

TTC was bought from Sigma-Aldrih Co. (St. Louis, USA). Total protein extraction kit and BCA protein quantization kit were bought from Ken Gen Biotech. Co. Ltd (Nanjing, China). Primary antibodies of cyt-c, Bcl-2, Bax, caspase-9, caspase-3, caspase-7, β-actin and GAPDH were from Cell Signaling Technology (Boston, USA). Secondary antibody of horseradish peroxidase-conjugated goat anti-rabbit IgG was from ZSGB-BIO (Beijing, China). Total RNA kit was from Tian Gen Biotech. Co. Ltd. (Beijing, China). Revertaid first strand cDNA synthesis kit was from Thermo Fisher Scientific Inc (New York, USA). SYBR® premix ex taq™ II (Tli RNaseH Plus) wasfrom Takara Bio Inc (Otsu, Japan).

2.3. Animals

Male SPF Sprague-Dawley (260–300 g) rats were purchased from Ningxia Medical University. The animals were maintained on a constant 12 h light/dark cycle at constant room temperature (23 ± 2 °C) and humidity (60%), they have free access to standard rodent food and water. Rats were divided into six groups and each group had twenty animals: Sham group, I/R group, nimodipine group (12 mg/kg, dissolved in 5% CMC-Na) and MXYF groups (58, 116 and 232 mg/kg, gastric gavages, respectively). Sham and I/R groups received 0.5% CMC-Na (10 ml/kg) solutions and treated groups received nimodipine and MXYF orally (10 ml/kg) for 3 days after induction of cerebral ischemia.

2.4. The model of cerebral I/R injury

Cerebral ischemia model was induced by middle cerebral artery occlusion for 2 h followed by 48 h reperfusion as described previously. Sprague-Dawley rats were anesthetized by intraperitoneal injection of 7% chloral hydrate (5 ml/kg). The right common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were exposed, the nylon wire (1.8 ± 0.5 cm) was advanced from CCA into ICA until it blocked the origin of the middle cerebral artery (MCA). Rats that showed excessive bleeding, dyspnea, subarachnoid hemorrhage and early death were excluded. Sham operated animals were subjected to the same surgical procedure, but without occlusion of the middle cerebral artery.

2.5. Measurement of neurological functional score

Neurological functional score was performed at 48 h after reperfusion (n = 8). Deficits were scored on a five-point scale adapted from a previous publication (Liang et al., 2011). Briefly, rats were scored as follows: 0-no observable neurological deficit; 1-failure to extend opposite forepaw; 2-circling to the contralateral side; 3-tumbled to its side while walking because of hemiplegia; 4-lost consciousness or died.

2.6. Measurement of cerebral infarct area

The rat brains were sliced into 2.0 mm sections. The brain slices were incubated in 2% triphenyltetrazoliumchloride for 30 min at 37 °C and then fixed in 10% formalin solution (n = 8). The infarct area showed white color while the normal brain tissue was in red color. The ratio of infarct area to total brain area was calculated.