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The natural therapeutic magnesium lithospermate B potently provides neuroprotective effects on cerebral ischemia/reperfusion injury in rats



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ABSTRACT

Ethnopharmacological relevance: *Salvia miltiorrhiza*, a perennial plant in the genus *Salvia* and popularly known as “Danshen”, is highly valued for its roots in traditional Chinese medicines (TCMs). It has widely used for the treatment of cerebrovascular and cardiovascular diseases in China. Recently, the cerebral protection of magnesium lithospermate B (MLB), a working extract from *Salvia miltiorrhiza*, has received more attention. Here, we investigated the therapeutic effects of MLB on cerebral ischemia/reperfusion (CI/R) injury using the middle cerebral artery occlusion (MCAO) model in rats.

Materials and methods: Male Sprague-Dawley rats were subjected to CI/R using a thread to occlude the right middle cerebral artery. After 2 h of cerebral ischemia, the middle cerebral artery was reperused for 24 h. Rats were injected with different doses of MLB (15, 30 and 60 mg/kg). Infarct zones, neurological deficit scores, brain water content, glutamate levels and protein expressions were evaluated after 24 h of reperfusion.

Results: We found that MLB treatment of rats exposed to focal CI/R decreased neurological deficit scores, brain water content, glutamate levels and cerebral infarct zones. We also demonstrated that MLB can inhibit CI/R injury-induced activation of caspase-3, a marker of apoptosis. This protection by MLB against CI/R injury was accompanied by an upregulation of p-Akt in the ischemic hemisphere. Furthermore, the MLB-induced protection was prevented by treatment with a PI3K inhibitor (LY-294002).

Conclusions: The data in the present study suggest a potential protective role of MLB against CI/R injury in rats. The salient finding of the present study is that this protective effect of MLB is likely mediated through an Akt-dependent pathway.

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1. Introduction

Stroke is the second most common cause of death and a major cause of disability around the world. Ischemic stroke occurs due to impairment of the blood supply to the brain or any of its parts, resulting in glucose and oxygen deprivation. Effective treatment of ischemic stroke requires re-canalization of occluded cerebral blood vessels. However, reperfusion after cerebral ischemia can cause brain

injury, leading to cerebral edema, brain hemorrhage, and neuronal death. This phenomenon is termed as cerebral ischemia/reperfusion (CI/R) injury (Donnan et al., 2008). However, few CI/R treatments have been successfully applied in clinical practice, although several strategies and drugs have been shown to decrease CI/R damage in animal models (Auriel and Bornstein, 2010; Sahota and Savitz, 2011). Recent studies have focused on the application of traditional Chinese medicines (TCMs) in the treatment of cerebral injury, as many TCMs and their active components are reported to possess neuroprotective efficacy. Indeed, Danhong injection[®] and ligustrazine hydrochloride injection[®] have been effective treatments for CI/R injury in China (He et al., 2012). As such, TCMs exhibit desirable effects on reperfusion injury, suggesting that they may be potential novel reperfusion injury therapies (Jia et al., 2008; Xuejiang et al., 1999). Thus, finding more effective therapeutic neuroprotective agents with minimal side

Abbreviations: CI/R, cerebral ischemia/reperfusion; MCAO, middle cerebral artery occlusion; MLB, magnesium lithospermate B; PI3K, phosphatidylinositol 3-kinase; TCMs, traditional Chinese medicines; TTC, 5-triphenyltetrazolium chloride

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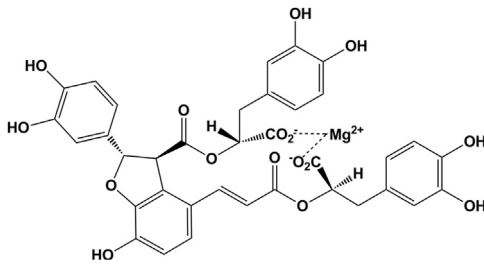


Fig. 1. Structure of MLB. Molecular formula: $C_{38}H_{28}MgO_{16}$; molecular weight: 740.90. MLB, magnesium lithospermate B.

effects from natural sources, such as herbs or plants, has become a priority in the field of TCMs.

Salvia miltiorrhiza, a perennial plant in the genus *Salvia* and popularly known as “Danshen”, is highly valued for its roots in TCMs. It has widely used in China and, to a lesser extent, in Japan, the United States, and European countries for the treatment of cerebrovascular and cardiovascular diseases. Magnesium lithospermate B (MLB, Fig. 1) is the active component in the water-soluble fraction of *Salvia miltiorrhiza* (Kim et al., 2010). Over the last decade, substantial scientific evidence has suggested that MLB has anti-fibrotic, myocardial protection, and neuroprotective effects (Tzen et al., 2007). MLB prevents hepatitis and uraemia, and it improves blood circulation, arrhythmia, and renal function (Fish et al., 2006; Lee et al., 2003). Recent work has shown that MLB possesses cardioprotective effects on myocardial ischemia/reperfusion injury, decreasing ST-segment elevation, reducing infarct size, and alleviating myocardial damage severity (Quan et al., 2013). However, up to now, few studies have considered the protective effects of MLB on CI/R injury. Thus, we assessed the potential benefit of MLB using the middle cerebral artery occlusion (MCAO) model in rats. In addition, one of the critical mechanisms of CI/R injury involves the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (Mullonkal and Toledo-Pereyra, 2007; Zhao et al., 2006). Extensive research has shown that the activation of PI3K/Akt during CI/R plays a pivotal role in reducing neuronal damage (Zhao et al., 2006). However, whether the activation of the PI3K/Akt pathway is involved in MLB-induced protection against CI/R injury remains unknown. Therefore, we further investigated the potential mechanism of MLB on CI/R injury, paying particular attention to the effects of MLB on the PI3K/Akt pathway.

2. Materials and methods

2.1. Ethics statement

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals, and all animal protocols were approved by the Fourth Military Medical University (FMMU) Committee on Animal Care. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Animals and chemicals

Adult male Sprague-Dawley rats (250 ± 20 g) were supplied by the animal research center at FMMU. Rats were housed individually under constant temperature (23 ± 3 °C) and humidity, with a 12-h light/dark cycle and free access to chow and water.

MLB was provided by Xi'an Honson Biotechnology Co. (Xi'an, China). Dried roots of *Salvia miltiorrhiza* plants cultivated in a local farm were prepared. The dried roots (8.8 kg) were extracted with 50 l methanol under reflux for 8 h and concentrated to a brown syrup. The syrup was suspended in H₂O and partitioned with

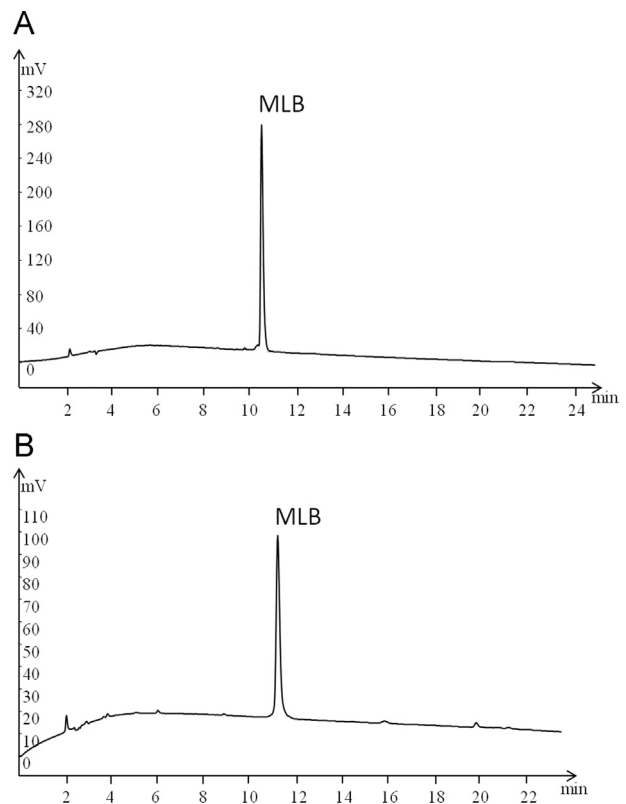


Fig. 2. Chromatograms of MLB. HPLC chromatographs showed the presence of MLB in the reference sample (A) and MLB provided by Xi'an Honson Biotechnology Co. (B). No significant differences were found between the retention time of MLB between (A) and (B) under the same HPLC conditions.

chloroform. MLB was harvested after repeated column chromatography of the H₂O extract using Sephadex LH-20 and H₂O as an eluent. High performance liquid chromatography (HPLC) analysis was performed. Briefly, the HPLC system (Agilent 1100 Series) consisted of a quaternary pump, C₁₈ gravity column (Alltima C₁₈, 250 × 4.6 mm, 5 μm) and DAD detector; mobile phase: acetonitrile–water–5% acetic acid = 21/78/1; flowrate: 1.0 ml/min; detector wave length: 286 nm (Fig. 2).

2,3,5-Triphenyltetrazolium chloride (TTC) and the PI3K inhibitor LY294002 (LY) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.). The BCA protein assay kit was obtained from Jiancheng Reagent Co. (Nanjing, China). All primary antibodies and goat anti-rabbit IgG secondary antibody were bought from Cell Signaling Co. (Cell Signaling, Santa Cruz, CA).

2.3. The MCAO model

Middle cerebral artery occlusion (MCAO) model was induced as previously described (Xu et al., 2006). After an overnight fast, rats were anesthetized by intraperitoneal injection of 40–45 mg/kg 2% (v/v) sodium pentobarbital, and placed in dorsal recumbency. Following a skin incision, the right common carotid artery, the external carotid artery (ECA), and the internal carotid artery (ICA) were exposed and carefully isolated. A 3-0 monofilament nylon suture was inserted from the lumen of the ECA to that of the right ICA to occlude the origin of the right middle cerebral artery. After 2 h of MCAO, the suture was carefully removed from the ICA. The neck incision was closed, and the rats were allowed to recover. During the surgical procedure, body temperature was maintained at 37 °C with a heating pad. The sham group underwent the same surgical procedure, except that the monofilament was introduced into the external carotid artery but not advanced.

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