

Neuroprotective effects of LBP on brain ischemic reperfusion neurodegeneration

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Abstract. – **AIM:** The present study was conducted to investigate whether LBP had a protective effect on cerebral ischemic reperfusion injury and to determine the possible mechanisms.

MATERIALS AND METHODS: Male Kunming (KM) mice were used to make the model cerebral artery occlusion/reperfusion (MCAO/R). The behavioral test was used to measure neurological deficit scores for evaluation of ischemic reperfusion damage of brain. The change of electroencephalograph (EEG) was monitored by Model SMUP-E Bio-electric Signals Processing System. The infarction area of brain was assessed in brain slices with 2% solution of 2,3,5-triphenyl tetrazolium chloride (TTC). Spectrophotometric assay was used to determine the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and lactate dehydrogenase (LDH), contents of malondialdehyde (MDA) and adenosine triphosphate (ATP) of the brain.

RESULTS: The results showed that LBP at doses of 20 and 40 mg/kg markedly decreased the neurological deficit scores and the infarction area in MCAO/R mice. At the same time, LBP significantly decreased MDA content, and increased SOD, GSH-Px, CAT, LDH activities in ischemic reperfusion brain.

CONCLUSIONS: These suggest that LBP might act as a potential neuroprotective agent against the cerebral reperfusion-induced injury in the brain through reducing lipid peroxides, scavenging free radicals, and improving the energy metabolism.

Key Words:

LBP, Focal cerebral ischemia/reperfusion, Neuroprotection, ROS.

Introduction

Acute ischemic stroke is the leading cause of adult disability and it is also an important cause of death in industrialized countries with high incidence affecting up to 0.2 of the population every year¹. Recent studies have provided direct and indirect experimental evidence that oxygen free radicals are elevated during ischemia reperfusion². The brain tissue with low activities of oxygen radical scavenging enzymes and high level of polyunsaturated fatty acids is particularly sensitive to cerebral ischemic reperfusion injury. Moreover, antioxidant defenses including free radical scavengers and antioxidant enzymes are limited to the extent of ischemic injury³. As a result of cerebral ischemic reperfusion, the occurrence of neurodegeneration is likely associated with changes in ATP⁴. As the overall process of ischemia reperfusion injury is extremely complex, the protective effects of medicinal herbs are receiving more attention in the effort to find agents for the treatment of ischemic cerebral vascular diseases.

The fruits of *Lycium barbarum* (also called Fructus Lycii, Wolfberry or Gouqizi), the small red berries, have been used for centuries in traditional medicine and cuisine. Accumulated studies showed that extracts from *Lycium barbarum* possess biological activities including anti-aging, anti-tumor, immune-stimulatory and cytoprotection^{5,6}. *Lycium barbarum* polysaccharide (LBP) is the major active ingredient of *Lycium barbarum*, Chan et al⁷ reported that the LBP

gave good neuroprotection to eyes. Many studies suggested that the protective effect of LBP mainly depends on its anti-oxidative action. For example, LBP⁸ can protect cultured seminiferous epithelium against hypothermia-induced damage by anti-oxidant mechanism. Another report⁹ showed that LBP can capture free radicals and restrain the DNA damage of testicle cells caused by the oxidative stress. However, it is not reported in the current literature whether LBP has a protective effect on cerebral ischemia reperfusion injury. Combining together, the present investigation was undertaken to evaluate the neuronal protective potential and possible mechanisms of LBP by detecting activities of oxygen radical scavenging enzymes and energy of metabolism in middle cerebral artery occlusion reperfusion (MCAO/R) induced focal cerebral ischemia-reperfusion model in rats.

Materials and Methods

Drugs and Reagents

LBP, brown powder with purity > 98%, was purchased from Ningxia Agricultural and Forestry College, and dissolved with Neurobasal-A-Medium (NAM). Nimodipine (NIM) (30 mg/piece), obtained from the Bayer Company (Germany). 2,3,5-triphenyl tetrazolium chloride (TTC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other commercial kits for the detection of lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and Glutathione peroxidase (GSH-PX) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were from commercial sources and of standard biochemical quality.

Animal Model and Design

Male KM mice weighing 25-30 g, from the Experimental Animal Center of Ningxia Medical University, were housed in cages of 5-6 at room temperature under a controlled 12 h light/dark cycle and allowed access to pellet food and water *ad libitum*. All experiments were performed as approved by the institutional animal care and use committee. Mice were randomly divided into six groups and each group had 13 animals. The first was the vehicle-treated group, that is, ischemia was induced for 2 h of MCAO followed by reperfusion for 24 h. The second was sham group. The LBP-treated groups were separated into a low

dosage group (LBP 10 mg/kg), a middle dosage group (LBP 20 mg/kg), and a high dosage group (LBP 40 mg/kg). The sixth was a nimodipine-treated group (0.4 mg/kg). The intragastric administration of LBP and nimodipine was conducted for five consecutive days and before MCAO/R. The mice focal cerebral ischemia reperfusion was produced by a modification of the monofilament method as described by Zea Longa et al¹⁰ and Macrae¹¹ except for mice in sham control group. Briefly, mice were anesthetized with 3.5% chloral hydrate in 0.9% NaCl (0.1 ml/10 g, i.p.) and placed in dorsal recumbency. Under sterile conditions, a ventral neck incision was made. The external carotid artery (ECA) and internal carotid artery (ICA) were exposed and carefully isolated. A nylon monofilament (15 mm in length and 0.15 mm in diameter) was inserted in the lumen of the left ECA and ICA to occlude the origin of the left MCAO. After 2 h of MCAO, the monofilament was removed to restore blood flow for 24 h reperfusion. The change of EEG was monitored by Model SMUP-E Bio-electric Signals Processing System (Department of Physiology and Pathology of Fudan University, Shanghai, China). Later, the neurological deficit score of each mouse was recorded on a 4-point scale¹² by a single experimenter, who was blinded to the different treatments by the experimenter in the experiment (no neurological deficit = 0, failure to extend right paw fully = 1, circling to right = 2, being unable to walk spontaneously and depression of consciousness = 3). Following the neurological evaluation, six mice from each group were decapitated to remove the brain. The occluded brain was cut into six slices of 1mm thick (Brain Matrices, Shenzhen, China), except the first slice was 2 mm and the brain slices were stained in 2% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) at 37°C for 30 min and then transferred into 4% formaldehyde solution for fixation. The normal brain tissue appeared uniform red while the infarction region showed white. The infarction volumes were calculated in a blinded manner with Adobe Photoshop CS 4 analysis system (Adobe Company, SAN Jose, CA, USA). The data was expressed as a percentage of the infarction volume/the ipsilateral hemisphere volume (%) at the coronal section of optic chiasma.

Determination of Adenosine Triphosphate (ATP)

24 h after reperfusion, whole brains were rapidly removed. Immediately after being

weighted and homogenized (10%,w/v) with cold 0.9% NaCl. ATP concentration was measured in accordance with the guide of the assay kit.

Monitor of Enzymes Activities and MDA Level

The ischemic hemisphere was homogenized in 1:9 (w/v) ice-cold salines after blotted and weighed to determine the level of malondialdehyde (MDA), activity of superoxide dismutase (SOD) and other indicators in ischemic brain. The homogenate was centrifuged at 3500 g and 4°C for 15 min, and then the supernatant was used to determine activity of GSH-Px, LDH, CAT, SOD and level of MDA with spectrophotometer (Beijing Rayleigh Analytical Instrument, BeiJing, China) as the methods provided by the assay kits (Nanjing Jianchen).

Statistical Analysis

SPSS16.0 software was used for statistical analysis (SPSS Inc., Chicago, IL, USA). Data were expressed as mean±standard error (±s). Multigroup comparison was made by analysis of variance and two-group comparison by *t*-test. Values were considered to be significant when *p* was less than 0.05.

Results

The results from Figure1 showed a significant EEG potential amplitude reduction and slow recovery with only 34.6% and 44.0% of which reverted to normal after 15 min and 90 min reperfusion respectively in the vehicle animals during cerebral ischemia. The cerebral ischemia-induced EEG changes were significantly promoted in each treatment group with remarkable EEG recoveries after reperfusion. After 15 min reperfusion, EEG potential amplitude was restored to 40.7%, 41.5% and 42.6% of normal in LBP 20, 40 mg/kg and nimodipine group respectively. EEG potential amplitude was restored to 54.4%, 85.9% and 75.4% of normal in the LBP 20, 40 mg/kg and nimodipine group respectively after 90 min reperfusion.

The findings from Figures 2, 3, 4 showed the neurological deficit score and infarction area of brain from different groups. The neurological deficit score and infarction area in the model control mice were significantly higher than those in the sham control mice. The treatment with 20, 40 mg/kg LBP and 0.4 mg/kg nimodipine signifi-

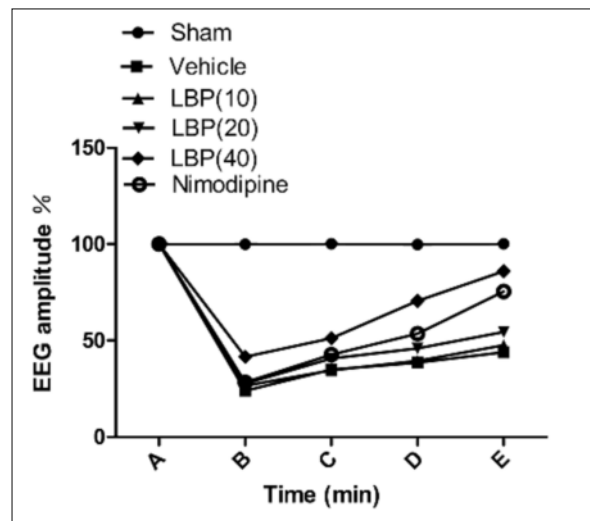


Figure 1. Effects of LBP on EEG during cerebral ischemia reperfusion in mice. A, B, C, D, E respectively is: before ischemia, 15 min after ischemia, 15 min after reperfusion, 45 min after reperfusion, 90 after reperfusion (n=6).

cantly reduced the infarction area and the neurological deficit score compared with the model control group (*p* < 0.05 or 0.01).

Table I showed that SOD and GSH-Px activities were much lower and MDA lever was higher in the model control group than those in the sham control group. The treatment with 10, 20,

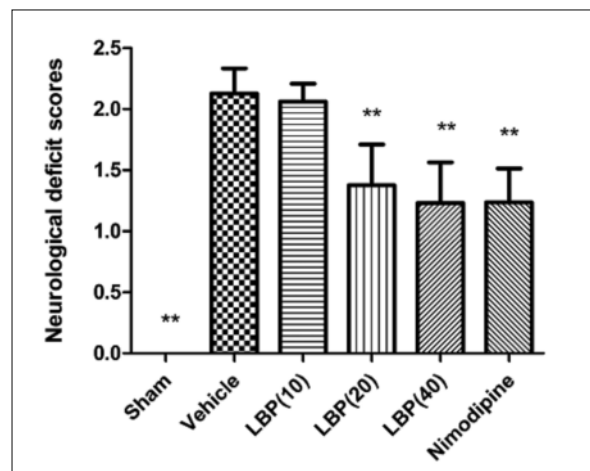


Figure 2. Effect of LBP on the neurological deficit score in MCAO/R mice. Animals were treated with LBP (10, 20, 40 mg/kg, i.g.), nimodipine (0.4 mg/kg, i.g.), or vehical (0.9% NaCl, i.g.) after the MCAO/R 24h, the neurological deficit score of mice was evaluated according to the Bederson's method. Data were presented as mean ± SD. One-way ANOVA test was used to determine statistical significance. Asterisks represent a significant difference model control group and drug-treated groups (**p* < 0.05; ***p* < 0.01).

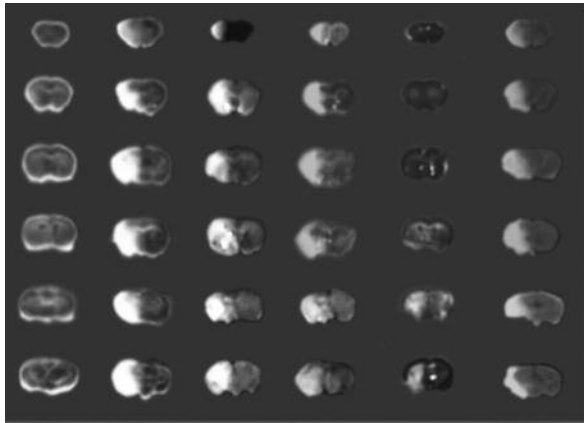


Figure 3. Infarction area in the cerebral hemisphere to show the effects of LBP and nimodipine on infarction area of brain after MCAO/R in mice. The order from left to right is sham control group, model control group, 10, 20, 40 mg/kg LBP groups, nimodipine group, respectively.

40 mg/kg LBP and 0.4 mg/kg nimodipine increased SOD and GSH-Px activities and decreased MDA content in brain tissue of the focal cerebral ischemic mice compared with the model control group ($p < 0.05$ or 0.01).

ATP content of brain mitochondria was quantified to investigate brain energy metabolism in cerebral ischemic reperfusion mice. Tables II showed that ATP level in the model control group were lower than that in the sham control. The treatments with 10, 20, 40 mg/kg LBP, or 0.4 mg/kg nimodipine significantly enhanced the ATP level compared with the control group.

Table II showed that LDH and CAT activities were much lower in the model control group than those in the sham control group. The treatment with 10, 20, 40 mg/kg LBP, or 0.4 mg/kg nimodipine increased LDH and CAT activities in

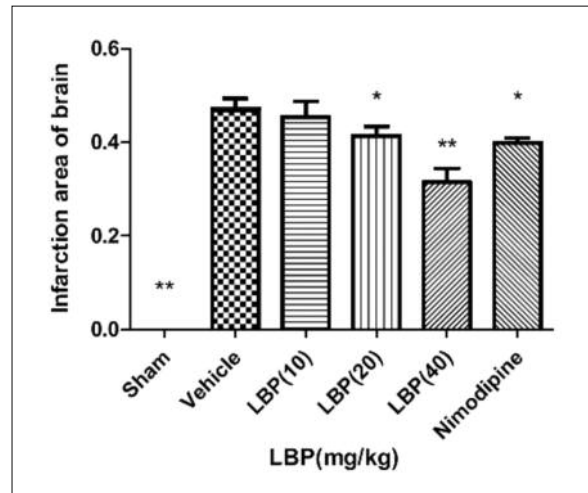


Figure 4. Effects of LBP on infarction area of brain in MCAO/R mice. Animals were treated with LBP (10, 20, 40 mg/kg, i.g.), nimodipine (0.4 mg/kg, i.g.), or vehical (0.9% NaCl, i.g.) after the MCAO/R 24h, the infarction area was quantified with method of TTC staining. Data were presented as mean ± SD (n=6). One-way ANOVA test was used to determine statistical significance. Asterisks represent a significant difference model control group and drug-treated groups (* $p < 0.05$; ** $p < 0.01$).

brain tissue of the focal cerebral ischemic mice compared with the model control group ($p < 0.05$ or 0.01).

Discussion

Many studies have shown that MCAO/R mainly induces hypoxic-ischemic damage in cerebral cortex. Cortical electrical activity decline is the first observed indicator in cerebral ischemia. EEG is often used as a monitoring indicator in cerebral ischemia and reperfusion injury as well

Table I. Effect of LBP on MDA level, SOD and GSH-PX activities in brain of mice after MCAO/R for 24h.

Groups	Number	Does (mg/kg)	MDA (nmol/mgprot)	SOD (U/mgprot)	GSH-Px (U/mgprot)
Sham	7	—	5.76 ± 0.88**	91.44 ± 3.46**	19.09 ± 1.23**
Vehicle	7	—	7.63 ± 0.84	40.65 ± 1.35	5.88 ± 1.03
LBP	7	10	8.23 ± 0.77	39.89 ± 4.16	7.42 ± 1.34*
	7	20	6.74 ± 0.96*	60.44 ± 3.22**	7.32 ± 0.77*
	7	40	4.85 ± 0.77**	75.36 ± 1.28**	13.37 ± 1.43**
Nimodipine	7	0.4	4.08 ± 0.74**	87.79 ± 4.17**	17.64 ± 1.11**

Mice in model and drug-treated groups were subjected to MCAO/R. Mice in sham group did not receive insertion of nylon monofilament and the rest procedures were same as model group. Animals were treated with LBP (10, 20, 40 mg/kg, i.g.), nimodipine (0.4 mg/kg, i.g.), or vehical (0.9% NaCl for sham and model groups, i.g.). After 2h the ischemia and reperfusion 24h, brains were quickly removed for determination of MDA content, SOD and GSH-PX activities with methods as described in assay kits. Data were presented as mean±SD. One-way ANOVA test was used to determine statistical significance. Asterisks represent a significant difference model control group and drug-treated groups (* $p < 0.05$; ** $p < 0.01$).

Table II. Effect of LBP on ATP content, CAT and LDH activities in brain of mice after MCAO/R for 24h

Groups	Number	Does (mg/kg)	LDH(U/gprot)	ATP (mmol/mgprot)	CAT (U/mgprot)
Sham	7	—	14025.35 ± 658.13**	17.50 ± 0.17**	30.76 ± 2.15**
Vehicle	7	—	7903.11 ± 487.35	6.21 ± 0.61	14.86 ± 1.39
LBP	7	10	7965.13 ± 464.25	6.60 ± 0.15*	15.53 ± 1.39
	7	20	9000.25 ± 618.81*	8.76 ± 0.62**	19.44 ± 1.19*
	7	40	10276.92 ± 524.15**	10.45 ± 0.24**	21.94 ± 2.21**
Nimodipine	7	0.4	12825.27 ± 661.23**	11.94 ± 0.75**	29.47 ± 1.88**

Animals were treated with LBP (10, 20, 40 mg/kg, i.g.), nimodipine (0.4 mg/kg, i.g.), or vehical (0.9% NaCl, i.g.). After ischemia 2h and reperfusion 24h, brains were quickly removed for determination of ATP content, LDH and CAT activities with methods as described in assay kits. Data were presented as mean ± SD. One-way ANOVA test was used to determine statistical significance. Asterisks represent a significant difference between model control group and drug-treated group (* $p < 0.05$; ** $p < 0.01$).

as evaluation of drug effects^{13,14}. We observed that LBP significantly improved the ischemia induced EEG changes and promoted the recovery of the EEG after reperfusion, which indicated that LBP possessed a satisfactory protective effect on cerebral ischemia reperfusion injury. From the electrophysiological aspect it hinted that LBP could improve the tolerability of brain cells lacking blood circulation.

The present study showed that LBP (i.g.) resulted in a significant dose-dependent reduction in neurological deficit and infarction area in the MCAO/R mice. A number of studies have proved that cerebral ischemia-reperfusion increases the content of reactive oxygen species (ROS) significantly and decreases the activity of antioxidant enzymes in the cerebral cortex. Reports show that antioxidant enzymes cannot efficiently eliminate ROS. Thereby, much ROS stored in a cell influences the normal activity of the cell. Many experiments show that ROS plays a role in the pathophysiology damage to membrane lipids and proteins¹⁵. The activities of antioxidant enzymes were decreased and the level of lipid peroxidation was increased significantly by MCAO/R and in the ischemic hemisphere in comparing with sham control in the experiment. LBP significantly prevented cerebral ischemia induced reduction of SOD, GSH-Px and CAT activities and elevation of MDA level in the brain tissue in a dose-dependent manner.

Previous studies have shown that ischemia impairs the metabolism of mitochondria¹⁶. MCAO/R induced ROS destroyed the membrane of mitochondria and influenced the function of respiratory chain so that ATP levels decreased significantly. As a result, cell initiates the process of apoptosis. LBP was able to increase ATP levels and protect neurons from ischemia-

reperfusion injury via enhancing activity of antioxidant enzymes activities and inhibiting ROS generation.

Ischemia causes calcium dysregulation leading to neuronal injury^{17,18}. As calcium channel blocker, Nimodipine is able to block calcium channel and maintain membrane stability effectively so as to alleviate brain injury induced by ischemia. It possesses an ability to reduce ROS and mitochondrial degeneration¹⁹. Findings from our study suggested that LBP possessed protective effect and hinted that it may be an important protective mechanism of LBP to upregulate activity of antioxidant enzymes and to decrease ROS release.

Conclusions

LBP is able to protect brain from the ischemic-reperfusion injury in mice and its mechanism may be partly attributed to scavenging free radicals by antioxidant enzymes and improving the energy metabolism of the brain. But, the precise mechanisms of LBP against focal cerebral ischemia need to be further studied.

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Conflict of Interest

There is no conflict of interest.

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