Astragalus Extract Alleviates Nerve Injury after Cerebral Ischemia by Improving Energy Metabolism and Inhibiting Apoptosis

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This aim of this study was to explore the effects and molecular mechanisms of Astragalus extract against cerebral ischemia injury through the energy metabolism and apoptosis pathways of c-Jun N-terminal kinase (JNK) signal transduction. After the bilateral common carotid artery of C57BL/6 mice was occluded for 20 min followed by 1-h reperfusion, the ATP content, total adenine nucleotides (TAN), energy charge (EC), and sodium potassium ATPase (Na⁺–K⁺-ATPase) activity were decreased markedly in brain tissues. Astragalus extract markedly increased the ATP and ADP levels, EC value, and Na⁺–K⁺-ATPase activity. Twenty-four and 48 h after reperfusion, the neurocyte survival rate decreased and apoptosis rate increased, while the expression of phosphorylated JNK1/2, cytochrome c (Cyt C), and cysteine aspartic acid-specific protease (caspase)-9 and -3 were significantly enhanced in brain tissues. Astragalus extract significantly increased neurocyte survival and decreased the apoptosis rate as well as down-regulated the expression of p-JNK1/2, Cyt C, caspase-9, and caspase-3. These results suggest that Astragalus extract has neuroprotective effects against nerve injury after cerebral ischemia-reperfusion, and the underlying mechanism may be associated with improved cellular energy metabolism, inhibition of JNK signal transduction pathway activation, and then suppression of the mitochondrial apoptosis pathway.

Key words Astragalus extract; cerebral ischemia-reperfusion injury; energy metabolism; apoptosis; phosphorylated c-Jun N-terminal kinase 1/2; cytochrome c

Experimental Animals Three hundred C57BL/6N male mice of clean grade, weighing 18–22 g, were provided by Hunan Slaccas Jingda Co., Ltd. (certificate of conformity: SCXK 2009-0004). Animals were allowed food and water ad libitum and were caged in a controlled environment with room temperature of 20–25°C and humidity of 50–60%. The animal protocols were approved by the Animal Ethics Committee of Hunan University of Chinese Medicine (approval number: HNCTCM 2008-085; date: June 17, 2008), and the disposal of animals during the experiments accorded with the "Guidance Suggestions for the Care and Use of Laboratory Animals" from the Ministry of Science and Technology, P. R. China (2006).

Drugs and Component Content Analysis Astragalus extract was provided by the Chengdu Hekang Pharmaceutical Co. (batch number: HK080302; Chengdu, P. R. China) and was extracted from the root of the leguminous plant Astragalus membranaceus (Fisch.) Bunge, and the original plants were stored in the herbarium of Chengdu Hekang Pharmaceutical Co. The glycoside components of Astragalus extract were determined to be 45% using chemical colorimetry at the Chengdu Branch of the Chinese Academy of Sciences. The astragaloside IV content of Astragalus extract was determined using type 2487 HPLC (Breeze work station, Waters

MATERIALS AND METHODS

This is a serious human health risk, and reperfusion plays an important role in cerebral ischemic injury. Along with further studies on cerebral ischemia-reperfusion injury, discovering effective agents for the prevention and treatment of ischemic cerebrovascular disease has become urgent. Astragalus is a common traditional Chinese medicine used for the treatment of cardiovascular and cerebrovascular disease. Astragalus extract contains polysaccharides and glycosides. Glycosides are the main active ingredient in Astragalus with cardiocerebrovascular pharmacological effects, and the main component is astragaloside IV (Fig. 1). Previous studies showed that Astragalus and its components may antagonize oxidative injury,¹,² protect neurons,³ and inhibit the increased blood–brain barrier (BBB) permeability⁴ after cerebral ischemia. However, it has not yet been determined whether Astragalus has antagonistic effects on energy metabolism disturbance and apoptosis of nerve cells after cerebral ischemia. This research was undertaken to study the effects and mechanisms of Astragalus extract against cerebral ischemia-reperfusion injury through the energy metabolism and apoptosis pathway of c-Jun N-terminal kinase (JNK) signal transduction, providing an experimental basis for the development of a clinical agent.

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Fig. 1. Chemical Structure of Astragaloside IV
Company, Massachusetts, U.S.A.). The reference substance of astragaloside IV (purity: 98.0%) was purchased from the Biological Products Analysis Bureau of the Ministry of Public Health, P.R. China. The chromatographic conditions were: chromatographic column, C18 (4.60 mm×250 mm, 5 μm); temperature, 35°C; flow rate, 1 mL/min; injection volume, 10μL; wavelength, 203 nm; and mobile phase, acetonitrile–water (33:67, v/v). The control solution (0.5 mg/mL) was prepared using the mobile phase, and the sample solution was prepared using Astragalus extract 1 g dissolved and filtered with 5 mL of the mobile phase before HPLC of the standard substance and sample (Fig. 2). Based on the chromatographic peak areas of the standard substance and sample, we calculated that Astragalus extract contained 38% astragaloside IV. Astragalus extract was prepared in a suspension with 0.5% sodium carboxymethylcellulose before use.

The positive control edaravone was provided by Nanjing Pharmaceutical Co., Ltd. (Nanjing, P.R. China) (purity: 99%), prepared in 0.4 mg/mL of solution with normal saline.

**Main Reagents** An ultramicro sodium potassium ATPase (Na⁺–K⁺-ATPase) assay kit and a total protein assay kit were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, P.R. China). ATP, ADP, and AMP standard substances (import subpacks) were purchased from Tianjin Yifang Co., Ltd. (Tianjin, P.R. China). Anti-mouse β-actin, phosphorylated JNK1/2 (p-JNK1/2), cytochrome c (Cyt C) monoclonal antibody, anti-mouse caspase-9 and caspase-3 polyclonal antibodies, and enhanced chemiluminescence reagent were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The corresponding peroxidase-labeled second antibodies were purchased from Beijing Sino-Shan Biotechnology Co., Ltd. (Beijing, P.R. China). A terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick-end labeling (TUNEL) apoptosis detection kit was purchased from Tianjin Hao Yang Biotech Co., Ltd.

**Animal Groups, Astragalus Extract Administration, and Model** According to reports in the literature,¹ Astragalus extract (containing 68% glycosides) 25, 50, and 100 mg/kg in mice or 20, 40, and 80 mg/kg in rats administered intragastrically had protective effects against cerebral ischemia-reperfusion injury. In addition, when Astragalus extract (containing polysaccharides and glycosides) was given as an intraperitoneal injection to mice, the maximal dosage (5000 mg/kg) showed no toxic effects, the median lethal dose (LD₅₀) could not be determined, and the maximum tolerable dose (MTD) mice was 5 g/kg.⁵ Therefore, we selected the Astragalus extract dose of 110 mg/kg, which was 1/45 of the MTD when administered via intraperitoneal injection in mice. Mice were randomly divided into the sham-operated, model, Astragalus extract (110 mg/kg/d, intragastrically), and edaravone (8 mg/kg/d, intraperitoneally) groups, with 5–10 mice in each group. The edaravone group received an intraperitoneal injection of edaravone (10 mL/kg) twice daily for 4 d. The sham-operated group and model control group were administered intragastric 0.5% sodium carboxymethyl cellulose (10 mL/kg), and the Astragalus extract group was given the same volume/weight by gavage at 08:00 once daily for 4 d. One hour after administration on day 4, the cerebral ischemia-reperfusion model was established following the method reported in the literature⁷ by occluding the bilateral common carotid artery (CCA) with an artery clip for 20 min. Mice in the sham-operated group underwent surgical exposure of the bilateral CCA. After suturing the skin, the mice continued to be medicated as above with rectal temperature maintained at 36.5–37.5°C on a heated laboratory table until awakening from anesthesia. At different times (1, 24, 48 h) after reperfusion, the mice were decapitated, the brain stem and cerebellar cortex were removed, and the brain tissues were examined.

**Detection of Energy Metabolism** After 1-h reperfusion, 60 mg of brain tissue was removed from the front of the optic chiasm and added to 5% cold perchloric acid (brain tissues: 5% perchloric acid: 1:9) to yield a 10% homogenate, which was centrifuged for 15 min at 4°C (15000 rpm). Then, 3 mol/L of K₂CO₃ (0.06 mL) was added to the supernatant (0.4 mL), the pH was adjusted to neutral with 10% NaHCO₃, and the mixture was centrifuged for 5 min (3000 rpm) to obtain the supernatant. ATP, ADP, and AMP levels were measured with HPLC.⁸ The total adenine nucleotides (TAN) and energy charge (EC) were calculated according to the following formula: TAN=[ATP]+[ADP]+[AMP]; EC=[(ATP)+0.5×[ADP]]/[ATP]+[ADP]+[AMP].⁹

Brain tissue was also removed from the posterior of the optic chiasm, added to cold saline (brain tissue:saline=1:9) to prepare a 10% homogenate, which was centrifuged for 5 min at 4°C (1000 rpm) to obtain the supernatant. Na⁺–K⁺ATPase activity was then evaluated in the phosphorus assay.

**Detection of Histomorphology in Hippocampal CA1** Twenty-four and 48 h after reperfusion, 1–4 mm of brain tissue (coronal slices, 5 μm) were removed from the posterior of the optic chiasm, stained with hematoxylin and eosin (HE), fixed in formalin, and embedded in paraffin. In 5 visual fields of a high-power microscope (×400), the survival rate of nerve cells was counted (percentage of normal nerve cells among total nerve cells). In normal nerve cells, the nucleus was blue, the cytoplasm was red, the cell membrane was clear, and nucleioli could be seen.

**Detection of Nerve Cell Apoptosis in Hippocampal CA1** Twenty-four and 48 h after reperfusion, 1–4 mm of brain tissue was dissected from the posterior of the optic chiasm (the remainder was used to detect protein expression), fixed with 10% paraformaldehyde, embedded in paraffin, and 7-μm coronal sections were used to determine the apoptosis rate in the TUNEL assay.¹⁰ Three nonoverlapping views were taken of
TABLE 1. Comparisons of Indicators of Energy Metabolism among Groups (n=10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>ATP (10^3 μg/mL)</th>
<th>ADP (10^3 μg/mL)</th>
<th>AMP (10^3 μg/mL)</th>
<th>TAN (10^3 μg/mL)</th>
<th>EC</th>
<th>Na^+-K^-ATPase (μmol/Pg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>29.1±3.5</td>
<td>26.2±5.2</td>
<td>59.6±8.9</td>
<td>112.0±8.5</td>
<td>0.37±0.04</td>
<td>4.45±1.49</td>
</tr>
<tr>
<td>Model</td>
<td>12.5±1.4</td>
<td>19.4±5.8</td>
<td>60.0±5.2</td>
<td>89.5±10.5</td>
<td>0.25±0.02</td>
<td>2.34±0.60</td>
</tr>
<tr>
<td>Astragalus extract</td>
<td>17.2±2.0</td>
<td>25.8±5.6</td>
<td>53.0±9.2</td>
<td>96.0±12.7</td>
<td>0.32±0.02</td>
<td>3.29±0.96</td>
</tr>
<tr>
<td>Edaravone</td>
<td>23.4±3.9</td>
<td>26.4±6.3</td>
<td>58.3±14.4</td>
<td>108.0±18.4</td>
<td>0.34±0.06</td>
<td>3.38±0.92</td>
</tr>
</tbody>
</table>

ATP, ADP, AMP contents were measured with HPLC, TAN=[ATP]+[ADP]+[AMP], EC=[(ATP]+[ADP]+[AMP])/[(ATP]+[ADP]+[AMP]], Na^+-K^-ATPase activity was evaluated by phosphorus (Pi) assay, data are presented as the mean±S.D. a) p<0.01, vs. sham-operated group; b) p<0.05, c) p<0.01 vs. model group; d) p<0.01, vs. Astragalus extract group.

each section at high magnification (×400). According to morphologic criteria, TUNEL-positive nuclei with brown granules were considered as apoptotic cells, and normal nuclei were blue. The apoptotic rate was then calculated as the percentage of apoptotic cells among total nerve cells in hippocampal CA1.

**Protein Expression of p-JNK1/2, Cyt C, Caspase-9, and Caspase-3 in Brain Tissue** One hundred milligrams of brain tissue was homogenized with 1 mL of cell lysate, the protein concentration was determined using the bicinchoninic acid method, and protein expression was detected with Western blotting. The X-ray film was placed in the image analysis system of Pro-Plug6.0 to determine the integral optical density (IOD) of the objective band, using β-actin as an internal reference and the ratio of the IOD values of the objective protein band and β-actin protein objective band as relative expression of the target protein.

**Statistical Analysis** The statistical software package SPSS 15.0 (SPSS Inc., Chicago, IL, U.S.A.) was used for all analyses. Data are expressed as mean±S.D. One-way analysis of variance (ANOVA) was used for multigroup pairwise comparisons, the least significant difference test was used for analysis of homogeneity of variance, and Dunnett’s T3 test was used for analysis of heterogeneity of variance. p values of less than 0.05 were considered to represent a statistically significant difference.

**RESULTS**

**Comparisons of Indicators of Energy Metabolism** After 1-h reperfusion the ATP content, TAN and EC values, and Na^+-K^-ATPase activity were obviously decreased in the model group compared with the sham-operated group (all p<0.01) (Table 1). Astragalus extract and edaravone markedly increased the ATP and ADP levels, EC value, and Na^+-K^-ATPase activity in the brain (p<0.05 and p<0.01, respectively). The TAN value showed a greater increase in the edaravone group, and the ATP level was higher than that in the Astragalus extract group (both p<0.01).

**Comparison of Histomorphology in Hippocampal CA1** Twenty-four and 48 h after reperfusion, 2 or 3 layers of pyramidal cells were seen in hippocampal CA1 in the sham-operated group (Figs. 3a–c). Those pyramidal cells were arranged closely and neatly, and the nuclei were large and round with 1 or 2 nucleoli. In the model group, pyramidal cells had lost their normal structure and were disorganized. Vacuolate degeneration and eosinophilic-like changes in nerve cells were seen in the cytoplasm, and many nuclei had become pyknotic (shrunken and dark). Compared with the sham-operated group, the survival rate of nerve cells decreased in the model group (p<0.01). At 24 h and 48 h after reperfusion, vacuolate degeneration, eosinophilic-like changes, and karyopyknosis of nerve cells were reduced in the Astragalus extract and edaravone groups compared with the model group, and survival rates increased (all p<0.01); the increased survival rate in the edaravone group was more marked than that in the Astragalus extract group (both p<0.01).

**Comparison of Apoptosis in Hippocampal CA1** At 24 h and 48 h after ischemia-reperfusion, there were scattered individual apoptotic cells in hippocampal CA1 in the sham-operated group. Numerous apoptotic cells were seen in the model group, and the apoptotic rate was significantly higher than that in the sham-operated group (both p<0.01) (Figs. 4a–c). Compared with the model group, the apoptotic rates were significantly decreased in the Astragalus extract group and edaravone group 24 h and 48 h after reperfusion (all p<0.01), and the decreased apoptotic rate in the edaravone group was more obvious than that in the Astragalus extract group (both p<0.01).

**Comparisons of p-JNK1/2, Cyt C, Caspase-9, and Caspase-3 Protein Expression in Brain Tissue** At 24 h and 48 h after ischemia-reperfusion, the expression of p-JNK1/2, Cyt C, caspase-9, and caspase-3 was significantly increased in the model group compared with the sham-operated group (all p<0.01) (Figs. 5a, b, 6a, b). Compared with the model group, p-JNK1/2, Cyt C, caspase-9, and caspase-3 protein expression was significantly inhibited in the Astragalus extract and edaravone groups (p<0.01, p<0.05, respectively). However, the differences between the Astragalus extract group and edaravone group were not statistically significant (all p>0.05).

**DISCUSSION**

According to the patterns of manifestation after ischemia, brain damage is divided into two phases. The first is acute damage in a series of reactions following cerebral ischemia, which lead to acute neurocyte death. The main pathophysiologic mechanisms include energy metabolism disturbance; calcium overload; the production of oxygen free radicals, nitric oxide, and neurotoxic excitatory amino acids; and destruction of the BBB. The second phase is delayed neuronal damage (DND). After cerebral ischemia-reperfusion, ischemia triggers the mechanisms of secondary injury, further damaging nerve cells. The main pathophysiologic mechanisms of DND are inflammatory reaction and apoptosis.

As the main energy source of brain tissue, ATP is oxidized via Na^+-K^-ATPase and releases energy, which plays important roles in maintaining the physiologic functions of...
Fig. 3. Histologic Changes in Hippocampal CA1 of Mice after Ischemia-Reperfusion

HE-stained sections of brain tissue showed normal morphology in the sham-operated group, while vacuolar degeneration, eosinophilic-like changes, and karyopyknosis of nerve cells were seen in the model group and treatment groups 24 h (a) and 48 h (b) after reperfusion (arrows) (×400, bar=20 µm). (c) Comparison of neurocyte survival rate in hippocampal CA1 24 and 48 h after reperfusion among groups. The survival rate of nerve cells was counted (percentage of normal nerve cells among total nerve cells), and data are presented as mean±S.D. (n=5). /p<0.01 vs. sham-operated group; /p<0.01 vs. model group; /p<0.01 vs. Astragalus extract group.

Fig. 4. Detection of Apoptotic Cells in Hippocampal CA1 24 h (a) and 48 (b) h after Reperfusion

Apoptotic cells were determined in the TUNEL assay, and TUNEL-positive nuclei with brown granules (arrows) were considered as apoptotic cells (×400, bar=20 µm). (c) Comparison of the apoptotic rate in hippocampal CA1 among groups 24 h and 48 h after reperfusion. The apoptotic rate was calculated as the percentage of apoptotic cells among total nerve cells, and data are presented as mean±S.D. (n=5). /p<0.01 vs. sham-operated group; /p<0.01 vs. model group; /p<0.01 vs. Astragalus extract group.
nerve cells such as active transport, protein synthesis and processing, ion homeostasis, cell membrane integrity, synaptic transmission, and neurotransmitter uptake and release.¹² The TAN level and EC value are important parameters that measure the metabolic status of the body, tissues, and cells. TAN reflects the oxidative respiratory activity of mitochondria and the ability to generate high-energy phosphate compounds. The cellular energy metabolism status is reflected by the EC, which is a dynamic parameter reflecting the cellular energy balance and can effectively assess cell energy reserves, indicating active generation of ATP if the EC value is high, or insufficient generation of ATP or increased ATP utilization. Na⁺–K⁺-ATPase depends on ATP to supply direct free energy. Studies showed that Na⁺–K⁺-ATPase activity is decreased after cerebral ischemia, which can lead to high Na⁺ influx, K⁺ outflow, cellular edema, and acute necrosis.¹³

Our study showed that the ATP level, TAN and EC values, and Na⁺–K⁺-ATPase activity clearly decreased after 1-h reperfusion. Neural cell viability decreased 24 h and 48 h after reperfusion in the model group, suggesting that cerebral ischemia-reperfusion resulted in a disruption of cerebral energy metabolism and nerve cell damage. Astragalus extract and edaravone increased ATP and ADP levels, the EC value, and Na⁺–K⁺-ATPase activity and attenuated nerve injury in the brain after cerebral ischemia, indicating that improving energy generation and supply may be one mechanism of action of Astragalus extract and edaravone against early neural damage.

JNK is an important signal transduction pathway involved in apoptosis.¹⁴,¹⁵ Activated JNK mediates apoptosis mainly by affecting gene expression (gene-dependent pathway) and the function of the mitochondrial (gene-independent pathway). In the gene-dependent pathway, JNK located in the cytoplasm immediately translocates into the nucleus after phosphorylation through the activation of c-Jun, Elk-1, activating transcription factor-2, etc., promoting the integration of activated transcription factors and cis-acting elements, inducing the expression of p53, Bax, FasL, tumor necrosis factor, and other proapoptotic proteins, thereby resulting in cell apoptosis, i.e., the nuclear pathway of apoptosis.¹⁶ In the mitochondrial pathway, JNK translocates near the mitochondrial permeability transition pore. Cyt C is then released into the cytoplasm, and cell apoptosis occurs through the expression and activation of caspase-9 and caspase-3.

Our results showed that cell apoptosis and the apoptosis rate increased markedly 24 h and 48 h after cerebral ischemia-reperfusion. Astragalus extract can attenuate nerve cell apoptosis, suggesting that it has an antiapoptotic effect after cerebral ischemia-reperfusion. The positive control edaravone is a free radical scavenger against oxidative damage and apoptosis, which was also confirmed in the present study. At the same time, the expression of p-JNK1/2, Cyt C, caspase-9, and caspase-3 was increased in the model group, suggesting that the JNK signal pathway was first phosphorylated and then the mitochondrial apoptosis pathway was activated, leading to apoptosis. Astragalus extract and edaravone can prevent those increases in protein expression, indicating that these agents have neuroprotective effects against apoptosis by suppressing JNK protein phosphorylation and mitochondrial apoptosis pathway activation after cerebral ischemia.

In conclusion, Astragalus extract alleviated nerve injury after cerebral ischemia. The two underlying mechanisms may be improving energy metabolism of brain tissue after cerebral ischemia-reperfusion. Neural cell viability decreased 24 h and 48 h after cerebral ischemia-reperfusion. The positive control edaravone increased ATP and ADP levels, the EC value, and Na⁺–K⁺-ATPase activity and attenuated nerve injury in the model group, suggesting that cerebra l ischemia-reperfusion resulted in a disruption of cerebral energy metabolism and nerve cell damage. Astragalus extract and edaravone increased ATP and ADP levels, the EC value, and Na⁺–K⁺-ATPase activity and attenuated nerve injury in the brain after cerebral ischemia, indicating that improving energy generation and supply may be one mechanism of action of Astragalus extract and edaravone against early neural damage.

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**Fig. 5. Western-Blot Patterns of Cyt C, Caspase-9, Caspase-3, and p-JNK1/2 Protein Expression 24 h (a) and 48 (b) h after Reperfusion.**

β-Actin was used as an internal reference.

**Fig. 6. Comparisons of p-JNK1/2, Cyt C, Caspase-9, and Caspase-3 Expression among Groups 24 h (a) and 48 h (b) after Reperfusion.**

Protein levels were measured using Western blot analysis, taking the ratio of the IOD values of the protein objective band and β-actin protein objective band as relative expression of target protein. Data are presented as mean±S.D. (n=5). ΔΔp<0.01 vs. sham-operated group; "p<0.05, "p<0.01 vs. model group.
ischemic, thus attenuating the early injury after cerebral ischemia-reperfusion, and inhibiting the mitochondrial apoptosis pathway of JNK signal transduction after cerebral ischemia, thus attenuating DND.

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REFERENCES