Effect of Ginkgo biloba leaf extract on cerebral cortex amino acid levels in cerebral ischemia model rats

Cui Yiran, Wu Hongwei, Liu Mengting, Qin Haijiao, Liu Xin, Yang Hongjun

Abstract

OBJECTIVE: To investigate the effect of Ginkgo biloba leaf extract on amino acid levels in the cerebral cortex of cerebral ischemia model rats induced by middle cerebral artery occlusion (MCAO).

METHODS: A rat model of cerebral ischemia was established by MCAO. Male rats were divided into a negative control group (Control), a sham-operated group (Sham), an ischemic group (MCAO), and an ischemic group treated with Ginkgo biloba leaf extract (MCAO_D). All groups were divided into two subgroups with occlusion times of 12 and 24 h, respectively. The levels of 18 endogenous amino acids in the cerebral cortex were quantified by triple quadrupole-liquid chromatography-mass spectrometry.

RESULTS: Compared with the MCAO group, behavioral performance, neurological deficit score, and cerebral infarct volume were significantly improved in the MCAO_D group (P < 0.05, P < 0.01). Compared with the sham group, the levels of 17 amino acids in the cerebral cortex were markedly changed in the MCAO group. The levels of Alanine (Ala), Isoleucine (Ile), Glutamic acid (Glu), Serine (Ser), Valine (Val), Phenylalanine (Phe), Proline (Pro), Threonine (Thr), Lysine (Lys), Tyrosine (Tyr), Hydroxyproline (Hyp), Arginine (Arg), Leucine (Leu), Tryptophan (Trp), and Glycine (Gly) were increased (P < 0.001, P < 0.05), while levels of Gln and Tau were decreased (P < 0.001, P < 0.05). Compared with the MCAO group, Ginkgo biloba extract treatment in the MCAO_D group significantly down-regulated the levels of 11 amino acids, especially those of Arg, Thr, and Ser in 12 or 24 h.

CONCLUSION: Injection of Ginkgo biloba leaf extract has a therapeutic effect on model rats with MCAO-induced cerebral ischemia by acting on amino acids in the cerebral cortex. This effect might be associated with the regulation of amino acid metabolism in the cerebral cortex.

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Keywords: Cerebral ischemia; Ginkgo biloba leaf extract; Amino acids; Metabolism

INTRODUCTION

Cerebral ischemic stroke, a leading cause of death and disability worldwide, is a severe neurological disorder that is usually induced by a temporary or permanent decrease in the blood supply to the brain. An ischemic...
stroke is a dynamic process in which perfusion or diffusion change throughout the evolution of the infarct. In the course of its occurrence and development, cerebral infarction is closely related to changes in the amino acid metabolism. Exploring the characteristics of amino acid metabolism can help identify biomarkers of the disease, discover targets for drug therapy, and explain mechanisms of drug treatment.

Ginkgo biloba seeds were first mentioned in Compendium of Materia Medica and are used against cough, asthma, enuresis, and alcohol misuse. Ginkgo biloba leaf extract has been widely used as a phytochemical and dietary supplement to improve conditions involving impaired blood circulation, for example, in the prevention and treatment of cardiovascular disease and cerebral insufficiency disorders, such as peripheral and cerebrovascular disorders, and ischemic and Alzheimer-type dementia. Ginkgo biloba leaf extract injection can affect the levels of amino acids in the cerebral cortex of cerebral ischemic rats. However, in this study, the number of amino acids examined was small and the time period of reperfusion was short. It is, therefore, necessary to further study the effect of Ginkgo biloba leaf extract on amino acid metabolism to comprehensively reveal its therapeutic characteristics. Thus, in this study, we established a rat model of cerebral ischemia by middle cerebral artery occlusion (MCAO), and explored the effect of Ginkgo biloba leaf extract on amino acid levels in the cerebral cortex.

MATERIALS AND METHODS

Animals

Seven-week-old male Sprague-Dawley (SD) rats specific-pathogen-free (SPF) with body weight of 240-260 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. [Beijing, China, License No. SCXK (Jing) 2012-0001]. All animals were kept in an SPF facility at (25 ± 2) °C under a normal light cycle with free access to normal diet and water. Animals were acclimatized for 3 d before use in experiments. This animal study was approved by the Institutional Animal Care and Use Committee of China, and institutional guidelines for animal welfare and experimental conduct were followed.

Drugs and reagents

The Ginkgo biloba leaf extract was made by Ji Sheng Medical Co., (Taipei, China Taiwan) and contained 17.50 mg of Ginkgo biloba leaf extract and 4.20 mg of ginkgo flavone glycosides (Production code HA0779). Standard references of alanine (Ala), isoleucine (Ile), glutamic acid (Glu), L-serine (Ser), valine (Val), L-phenylalanine (Phe), proline (Pro), L-threonine (Thr), hydroxyproline (Hypro), L-arginine (Arg), tryptophan (Trp), glycine (Gly), lysine (Lys), tyrosine (Tyr), leucine (Leu), glutamine (Gln), taurine (Tau), and γ-aminobutyric acid (GABA) were purchased from Sigma (USA). Other reagents used include, chloral hydrate (Sinopharm Chemical Reagent Co., Ltd.); 2,3,5-triphenyltetrazolium chloride (TTC) (Nanjing Green syn-chemical Technology Co., Ltd., Nanjing, China); 0.9% NaCl injection solution (Shijiazhuang siyao Co., Ltd., No. 1409143203, Shijiazhuang, China); 4% polyoxymethylene (Beijing Solarbio Science & Technology Co., Ltd., No. 20150424, Beijing, China).

Model building and drug administration

Male SD rats were randomly divided into eight groups: 12 h-control, sham, MCAO, and MCAO_D groups and 24 h-control, sham, MCAO, and MCAO_D groups. Twenty-three rats were allocated to each group. Focal cerebral ischemia was induced by intra-luminal MCAO as described previously. Briefly, rats were anesthetized and placed on a heating pad to maintain rectal temperature at 37 °C. A nylon filament was advanced from the right external carotid artery into the lumen of the internal carotid artery to occlude the right MCA for 12 h (12 h groups) or 24 h (24 h groups). The filament was subsequently withdrawn to allow reperfusion. Cerebral blood flow was closely monitored by laser Doppler flowmetry during the operation. Sham-treated rats were subjected to the same surgical procedure without the insertion of the nylon filament. For the 12 h MCAO_D group, rats were intraperitoneally injected with Ginkgo biloba leaf extract at 1.575 mg/kg (equivalent to a clinical dose) at 15 min and 6 h after modeling. The drug was prepared at a concentration of 11.25 mg/ml in 0.9% NaCl before use. For the 24 h MCAO_D group, rats were intraperitoneally injected with Ginkgo biloba leaf extract at 1.575 mg/kg at 15 min, 6 h, and 12 h after modeling. The other groups (control, sham, MCAO) were given 0.9% NaCl by intraperitoneal injection. The volume of all injections was 14.4 mL/kg.

Evaluation of neurological deficit

Before killing animals, Bederson’s neurological examination scoring system was used to evaluate neurological deficit in animals. Scoring standard: 0: no observable deficit and normal activity (normal); 1: not fully extending the left forepaw (mild deficit); 2: circling towards the contralateral side (moderate deficit); 3: unable to walk and righting reflex impairment (severe deficit).

Measurement of cerebral infarction by TTC staining

After the final evaluation of neurological deficit, eight rats from each group were sacrificed and the brains were removed. The cerebellum, olfactory bulb and the remainder of the lower brain stem were removed and a 2-mm thick brain slice at the intersection of the optic nerves was rapidly dissected. Slices were fixed in 4% paraformaldehyde for 30 min, and stained in 2% TTC in phosphate buffer for 10 min at 37 °C without light.
Determination of amino acids in rat cortex by LC-MS

An Agilent 6460 triple quadrupole (QQQ)-liquid chromatography-mass spectrometry (LC-MS) system (Agilent Technologies, Palo Alto, CA, USA) was utilized to determine amino acid levels. High pressure liquid chromatography was performed using an Agilent 1260 uHPLC system (Agilent Technologies, Palo Alto, CA, USA). The mass analyzer used was an Agilent 6460 triple quadrupole with an ESI interface (ESI-QQQ/MS, Agilent Technologies, Palo Alto, CA, USA).

Brain cortex tissue samples were weighted, and added with 10 times volume (1 g/10 mL) of different concentrations of methanol (20%, 50%, 100%). The tissue was then homogenized using a high throughput homogenizer (CIENIZ SC VT2-48, Ningbo Xingzhi Biotechnology Co., Ltd., Ningbo, China) at 70 Hz for 45 s. Twenty microliters of grinding fluid was added to 980 μL initial mobile phase. After vortexing for 1 min, the sample was centrifuged at 13,300 × g for 5 min at 4 °C and then the supernatant was collected and 5 μL of the supernatant were injected into the LC-MS system. According to the detection of amino acids by mass spectrometry, we selected the best extraction solvent (methanol) concentration. To select the volume of extraction solvent, we added different volumes of solvent (5, 10, 20, 50 times) to Control and MCAO groups samples, and detected the amino acid response intensity. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with electrospray ionization (ESI) either in positive or negative ionization mode. Amino acids were separated by an ACQUITY UPLC BEH Amide column (100 mm × 2.1 mm, 1.7 μm). Gradient elution was performed using water containing 0.1% formic acid-water (Solvent A) and acetonitrile containing 0.1% formic acid and 1% ammonium formate (0.1 mol/L) (Solvent B). Ten percent Solvent B (10%) was employed for 1 min and was increased to 40% B for 7 min at a flow rate of 0.25 mL/min. The 10% B was used for 2 min to re-equilibrate. Mass analysis of compounds was performed using positive ion mode. Other parameters were: nebulizer gas flow, 45.0 psi; dry gas flow, 11.0 L/min; electrospray voltage of the ion source, 4000 V; 52 nA; capillary temperature, 350 °C ; Collision Induced Dissociation (CID), 32 eV.

The external standard method was used to establish a methodology, including inter-and intra-day precision, linearity range, repeatability, stability and accuracy of sample determination. The linearity of 18 amino acids was determined by analysis of standard solutions of different concentrations. The intra-day and inter-day precision was determined by the assay of the standard solution at different concentrations on a single day and on three continuous days. Five determinations of each amino acid concentration were analyzed and precision and accuracy were expressed as relative standard deviation (RSD %) and deviation from the theoretical value. The stability of amino acids in cortex tissue was tested by analysis of five samples stored in 4 °C for 36 h. To determine the recovery, three samples were prepared and analyzed for amino acid content. Recovery of amino acids was calculated using the following equation: recovery = [(measured content-sample content)/addition content] × 100%.

Statistical analysis

SIMCA-P (version 11.5, Umetrics, Umeå, Sweden) software was used to carry out supervised pattern recognition analysis by partial least squares discriminate analysis (PLS-DA). The differences between the sham and MCAO groups, and between MCAO and MCAO_D groups were observed. Metabolic markers between the groups were screened by variable importance in the projection values (VIP) (VIP > 1). Data are expressed as mean ± standard error of mean (SEM) or as percentages and were analyzed for statistical significance using one-way analysis of variance. Tests were performed using SPSS 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0, Armonk, NY, USA); a P value less than or equal to 0.05 was considered statistically significant.

RESULTS

Effects of Ginkgo biloba leaf extract on MCAO-induced cerebral ischemia

The effects of Ginkgo biloba leaf extract on neurological function scores after cerebral ischemia are shown in Figure 1A. These two indicators of neurological behavior and cerebral infarct in control and sham group were zero. Compared with the sham group, neurological behavior was strongly affected in the 12 and 24 h MCAO groups, as indicated by neurological scores measured (P < 0.001). Significant improvements in neurological scores were observed in the 24 h MCAO_D group rats treated with Ginkgo biloba leaf extract compared with the 24 h MCAO group (P < 0.05). The cerebral infarct area and volume were also reduced in drug-treated rats (Figure 1B and Figure 2).

Determination of 18 amino acids in brain cortex tissue

Brain tissue was extracted with methanol at a ratio of 1:20. To optimize the methanol concentration for extraction, 20%-100% methanol was used to extract
The normal tissue stained a rose red color, and the infarct tissue was white.
and Tau were significantly decreased ($P < 0.001$, $P < 0.05$). For the 12 h groups, Ginkgo biloba leaf extract treatment in the MCAO_D group significantly decreased the levels of Ala, Ser, Gln, Thr, Arg and Tau ($P < 0.001$, $P < 0.01$, $P < 0.05$) and increased the levels of Phe and Tyr ($P < 0.001$, $P < 0.05$) compared with the MACO group. In the 24 h groups, Ginkgo biloba leaf extract treatment in the MCAO_D group significantly reduced the levels of Ile, Ser, Phe, Pro, Thr, Lys, Tyr, Tau, Hyp, Arg, Leu and Gly compared with the MACO group ($P < 0.001$, $P < 0.01$, $P < 0.05$) (Figures 3-4).

PLS-DA was used to analyze the influence of amino acid levels in each group. A PLS-DA discriminant model was constructed to analyze the amino acid levels in the sham, MACO and MACO_D groups (Q2 value: 12 h groups, 0.934, and 24 h groups, 0.696). The PLS-DA score map showed that the sham, MACO and MACO_D groups were separated, indicating that levels of the 18 amino acids changed significantly during the process of cerebral ischemia (Figure 5). At the same time, the amino acids with a VIP value >1 in the MACO_D group at different time points were selected and compared with those of the MACO group. There were seven distinct amino acids in the 12 h groups and nine in the 24 h groups (Table 3).

Table 1 Monitoring ions of various amino acids in MRM mode

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Parention</th>
<th>Fragmentation</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Ala</td>
<td>90.07</td>
<td>44.27</td>
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<tr>
<td>2</td>
<td>γ-GABA</td>
<td>104.09</td>
<td>87.01</td>
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<tr>
<td>3</td>
<td>Ile</td>
<td>132.12</td>
<td>86.16</td>
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<tr>
<td>4</td>
<td>Glu</td>
<td>148.08</td>
<td>84.07</td>
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<tr>
<td>5</td>
<td>Ser</td>
<td>106.01</td>
<td>60.10</td>
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<tr>
<td>6</td>
<td>Val</td>
<td>118.00</td>
<td>71.90</td>
</tr>
<tr>
<td>7</td>
<td>Phe</td>
<td>166.11</td>
<td>120.07</td>
</tr>
<tr>
<td>8</td>
<td>Gln</td>
<td>147.04</td>
<td>84.07</td>
</tr>
<tr>
<td>9</td>
<td>Pro</td>
<td>116.09</td>
<td>70.11</td>
</tr>
</tbody>
</table>

Table 2 Calibration curves of various amino acids

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Range (pg/μL)</th>
<th>calibration curves</th>
<th>r value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ala</td>
<td>10-300</td>
<td>$Y = 57.384X-1803.36$</td>
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<td>2</td>
<td>γ-GABA</td>
<td>10-300</td>
<td>$Y = 578.654X-3470.35$</td>
<td>0.996</td>
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<tr>
<td>3</td>
<td>Ile</td>
<td>1-30</td>
<td>$Y = 5897.31X+408.95$</td>
<td>0.992</td>
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<tr>
<td>4</td>
<td>Glu</td>
<td>30-1000</td>
<td>$Y = 3532.66X-1192.27$</td>
<td>0.999</td>
</tr>
<tr>
<td>5</td>
<td>Ser</td>
<td>10-300</td>
<td>$Y = 654.99X+2613.2$</td>
<td>0.999</td>
</tr>
<tr>
<td>6</td>
<td>Val</td>
<td>5-100</td>
<td>$Y = 2015.06X+450.45$</td>
<td>0.999</td>
</tr>
<tr>
<td>7</td>
<td>Phe</td>
<td>1.5-50</td>
<td>$Y = 15907X-110.09$</td>
<td>0.999</td>
</tr>
<tr>
<td>8</td>
<td>Gln</td>
<td>15-500</td>
<td>$Y = 34481.2X-588028$</td>
<td>0.967</td>
</tr>
<tr>
<td>9</td>
<td>Pro</td>
<td>5-100</td>
<td>$Y = 5047.65X+6909.01$</td>
<td>0.885</td>
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<tr>
<td>10</td>
<td>Thr</td>
<td>10-300</td>
<td>$Y = 2387.16X+146750$</td>
<td>0.996</td>
</tr>
<tr>
<td>11</td>
<td>Lys</td>
<td>10-300</td>
<td>$Y = 2453.57X+118549$</td>
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</tr>
<tr>
<td>12</td>
<td>Tyr</td>
<td>0.3-10.0</td>
<td>$Y = 2155.66X+16868.4$</td>
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</tr>
<tr>
<td>13</td>
<td>Tau</td>
<td>30-1000</td>
<td>$Y = 602.90X+2814.95$</td>
<td>0.996</td>
</tr>
<tr>
<td>14</td>
<td>Hyp</td>
<td>0.3-10.0</td>
<td>$Y = 6345.06X-290.138$</td>
<td>0.999</td>
</tr>
<tr>
<td>15</td>
<td>Arg</td>
<td>5-100</td>
<td>$Y = 6624.64X-3314.27$</td>
<td>0.993</td>
</tr>
<tr>
<td>16</td>
<td>Leu</td>
<td>5-100</td>
<td>$Y = 4940.3X+1094.83$</td>
<td>0.999</td>
</tr>
<tr>
<td>17</td>
<td>Trp</td>
<td>10-150</td>
<td>$Y = 4009.86X+913.613$</td>
<td>0.997</td>
</tr>
<tr>
<td>18</td>
<td>Gly</td>
<td>1-30</td>
<td>$Y = 4846.25X+1015.69$</td>
<td>0.996</td>
</tr>
</tbody>
</table>

Notes: Ala: alanine; Ile: isoleucine; Glu: glutamic acid; Ser: L-serine; Val: valine; Phe: L-phenylalanine; Pro: proline; Thr: L-threonine; Hyp: hydroxyproline; Arg: L-arginine; Trp: tryptophan; Gly: glycine; Lys: lysine; Tyr: tyrosine; Leu: leucine; Gln: glutamine; Tau: taurine; GABA: γ-aminobutyric acid.
PLS-DA analysis and statistical analysis between different groups were compared. By analyzing the VIP > 1 intersection at different times, it was found that five amino acids, Phe, Tau, Arg, Thr and Ser, were changed in the 12 and 24 h MACO groups. Differences between MACO and MACO_D groups gave a comprehensive analysis and showed that Ginkgo biloba leaf extract treatment had corrective action on Arg, Thr and Ser at 12 and 24 h, and in Phe at 24 h, with no corrective action on Tau.

**DISCUSSION**

In this study, we investigated the effect of Ginkgo biloba leaf extract on the metabolism of 18 amino acids in the rat cerebral cortex after cerebral ischemia for 12 and 24 h. The levels of Ala, Ile, Glu, Ser, Val, Phe, Pro, Thr, Lys, Tyr, Hyp, Arg, Leu and Gly were significantly increased ($P < 0.05$), while Gln and Tau were remarkably decreased in the MACO group compared with the sham group. The changing levels of Ala, Glu, Gly, Phe, Tyr, Thr, Val, Ile, Leu and Lys are consistent with those from previous reports. Ginkgo biloba leaf extract can reduce the levels of Ala, Ser, Val, Phe, Pro, Thr, Lys, Tyr, Hyp, Arg, Leu and Gly in brain cortex. A comprehensive analysis of the results by VIP value > 1 combined with multiple groups of variance analysis found that Ginkgo biloba leaf extract has a corrective action on Arg, Thr...
and Ser in the 12 and 24 h groups and on Phe in the 24 h group, while no corrective effect was observed for Tau. These results indicate that its effect on Arg, Thr and Ser in the cerebral cortex of rats with cerebral isch-

emia is persistent and stable.

When astrocytes are damaged, L-serine from glucose catabolism is transformed into D-serine at the presynaptic membrane, and D-serine is a coactivator of the
N-methyl-D-aspartate receptor (NMDAR). After exocytosis, D-serine activates NMDARs in the postsynaptic membrane, which causes calcium overload resulting in damage. Arg is a nitric oxide donor, which is generated by nitric oxide synthase. Nitric oxide is a signaling molecule that can regulate cellular function and is involved in cardiovascular system, and peripheral and central nervous system physiology. Nitric oxide has a dual role in the process of ischemic brain injury, indicating that the concentration of L-Arg may play a dual role in ischemic injury. Thr deficiency can inhibit the production of immunoglobulins and T and B lymphocytes, which then affects immune function. In conclusion, Ginkgo biloba leaf extract may improve the production of immunoglobulins and T and B lymphocytes, which then affects immune function. This study was supported by the Key Program of National Natural Science Foundation of China (81330086), the General Program of National Natural Science Foundation of China (81573726, 81403210) and the Fundamental Research Funds for the Central Universities (No. 2017-JYB-JS-054).

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REFERENCES

14 Mathers DA, McCarthy SM, Cooke JE, Ghavamini AA,


