Effects of the main active components combinations of *Astragalus* and *Panax notoginseng* on energy metabolism in brain tissues after cerebral ischemia-reperfusion in mice

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ABSTRACT

Background: *Astragalus* and *Panax notoginseng* are traditional Chinese medicines used for the treatments of cardio-cerebrovascular ischemic diseases, astragaloside IV (AST IV) and ginsenoside Rg1 (Rg1), ginsenoside Rb1 (Rb1), notoginsenoside R1 (R1) are their active components. **Objective:** The purpose of this work was to investigate the effect of AST IV combined with Rg1, Rb1, R1 on energy metabolism in brain tissues after cerebral ischemia-reperfusion in mice. **Materials and Methods:** C57BL/6 mice were randomly divided into 11 groups, treated for 3 days. At 1 h after the last administration, the model of cerebral ischemia-reperfusion injury was established, and brain tissues were detected. **Results:** All drugs increased the contents of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and the level of total adenine nucleotides (TAN), the combinations increased energy charge (EC), the effects of four active components combination were better. The phosphorylation of AMP-activated protein kinaseα1/2 (p-AMPKα1/2) was increased in AST IV, R1, four active components combination, AST IV + Rg1 and AST IV + R1 groups, the increased effect of four active components combination was greater than that of the active components alone and AST IV + Rb1. All drugs increased glucose transporter 3 (GLUT3) mRNA and protein, and the increases of four active components combination were more obvious than those of the active components alone or some two active components combinations. **Conclusion:** Four active components combination of *Astragalus* and *P. notoginseng* have the potentiation on improving of energy metabolism, the mechanism underlying might be associated with promoting the activation of AMPKα1/2, enhancing the expression of GLUT3, thus mediating glucose into nerve cells, increasing the supply and intake of glucose.

Key words: Active components combinations, AMP-activated protein kinase, *Astragalus* and *Panax notoginseng*, cerebral ischemia, energy metabolism, glucose transporter 3

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INTRODUCTION

Our previous studies show that astragalosides (AST) and *Panax notoginseng* saponins (PNS), the effective ingredients of *Astragalus* and *P. notoginseng*, have antagonistic effects on cerebral ischemia-reperfusion injury through multiple pathological links,[1,2] AST combined with PNS enhance the effects, which are related to inhibit the anti-oxidative stress injury and protect the blood-brain barrier.[3,4] As AST IV and ginsenoside Rg1 (Rg1), ginsenoside Rb1 (Rb1), notoginsenoside R1 (R1) are the active components of AST and PNS, and our previous studies have proved that AST IV, Rg1, Rb1, and R1 can relieve the nerve cells damage in hippocampus after cerebral ischemia-reperfusion, which is strengthened by the combination of the active components, the mechanism is derived from the antagonism on oxidative stress injury.[5] Thus, this study is to further probe the effect of AST IV combined with Rg1, Rb1, and R1 against cerebral ischemia-reperfusion injury from the perspective of energy metabolism disorder.

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MATERIALS AND METHODS

Experimental animal

C57BL/6 male mice, weighing 18–22 g, were provided by Hunan Vital River Laboratory Animal Technology Co., Ltd, (certificate of conformity: SCXK 2009-0004). Animals were housed four per cage in rooms maintained at 20~25°C with alternating 12 h light-dark cycle. Food and water were provided *ad libitum* throughout the experiments. The animal protocols were approved by Animal Ethics Committee of Hunan University of Traditional Chinese Medicine (approval number: HNCTCM 2011-085, date: 17-June-2011) and the disposal of animals during the experiment accorded with "guidance suggestions for the care and use of laboratory animals" from the Ministry of Science and Technology of the People's Republic of China.

Drugs and main reagents

AST IV (Batch number: A0070), Rg1 (Batch number: A0237), Rb1 (Batch number: A0234) and R1 (Batch number: A0273) were purchased from Chengdu Must Co., Ltd, China, purity ≥98%, prepared into the suspension with 0.5% sodium carboxymethyl cellulose before use; edaravone (3-Methyl-1-phenyl-2-pyrazolin-5-one) improved energy metabolism of tissues and antagonized oxidative stress injury,[6] as a positive control in this study, being provided by Nanjing Pharmaceutical Co., Ltd, China (Batch number: 80-090104, Specs: 10 mg/5 mL) and prepared into 0.4 mg/mL of solution with normal saline. Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) standard substances (Batch number: 08/2012) were purchased from Tianjin Yifang Co., Ltd, China; Total protein extraction kit (Batch number: KGP2100) was purchased from Nanjing KeyGen Biotech Co., Ltd, China; phosphatase inhibitor complex III (Batch number: PL019-1), modified bicinchoninic acid (BCA) protein assay kit (Batch number: SK3051) were purchased from Shanghai Sangon Biotech Co., Ltd, China; horseradish peroxidase (HRP) labeled goat anti-rabbit immunoglobulin G (IgG) was purchased from Beijing JinShan Biotech Co., Ltd, China; Tissue total RNA extraction kit (Batch number: Lo423) and enhanced HRP-diaminobenzidine (DAB) Substrate Chromogenic Kit (Batch number: L1026) were purchased from Beijing TianGen Biotech Co., Ltd, China; reverse Transcription kit (Batch number: ADA3500) was purchased from Promega (Madison, Wisconsin, USA), rabbit anti-mouse β-actin antibody (Batch number: sc-130656), phosphorylation of AMP-activated protein kinaseα1/2 (p-AMPKα1/2) (Batch number: SC-33524), glucose transporter 3 (GLUT3) antibody (Batch number: SC-30107) were purchased from Santa Gruz Biotech Co., Ltd, (California, USA).

Animal groups and administration

Based on the previous study,[5] the mice were randomly (digital random method) divided into 11 groups: Sham, control, AST IV (40 mg/kg), Rg1 (50 mg/kg), Rb1 (40 mg/kg), R1 (10 mg/kg), four active components combination (AST IV 40 mg/kg + Rg1 50 mg/kg + Rb1 40 mg/kg + R1 10 mg/kg), AST IV 40 mg/kg + Rg1 50 mg/kg, AST IV 40 mg/kg + Rb1 40 mg/kg, AST IV 40 mg/kg + R1 10 mg/kg and edaravone (4 mg/kg), with eight mice in each group. Edaravone group were received intraperitoneal injection of edaravone (10 mL/kg), twice daily for 3 days. Sham group and control group were gavaged with 0.5% sodium carboxymethyl cellulose (10 mL/kg), the left were given the same volume/weight medicine respectively by gavage at 8:00 am, once a day for 3 days. After the treatment for 1 h on day 3, the cerebral ischemia-reperfusion model was established. The same administration was carried out as above during reperfusion.

Model of cerebral ischemia-reperfusion

The cerebral ischemia-reperfusion model was established according to the literature.[5,7] The mice were fixed in supine position after anesthesia with ether, received 1 cm incision in the middle of the neck, exposed and separated the common carotid artery (CCA) and the accompanies vagus nerve, then, bilateral CCA were occluded with artery clip for 20 min, followed by reperfusion for 24 h. Mice in the sham-operated group only underwent surgical exposure of the bilateral CCA. Sutured the skin, the mice continued to be fed and medicated as above with body temperature maintained at 37–38°C. At 24 h after reperfusion, the mice were decapitated, the brain stem and cerebellar cortex were cleared away, and the remaining brain tissues were detected.

Detections of adenylate in brain tissues

Processing of tissue samples

To take 120 mg brain tissues from the front of right optic chiasm to prepare into 10% homogenate with 5% cold perchloric acid (brain tissues: 5% perchloric acid = 1:9), which was centrifuged for 15 min at 4°C (15,000 rpm) to acquire the supernatant; Then, the supernatant was added into 3 mol/L K_2CO_3 (0.06 mL), adjusted to pH 7 with 10% $NaHCO_3$, and centrifuged for 5 min (3000 rpm) at 4°C again. To detect the contents of ATP, ADP, AMP by high performance liquid chromatography (HPLC),[8] the values of 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]).

Preparations of adenosine triphosphate, adenosine diphosphate, adenosine monophosphate control mixture

To weigh precisely standard substance ATP 16.4 mg, ADP 15.0 mg, AMP 13.1 mg, which was dissolved with mobile phase to obtain the control mixture with concentrations of ATP 328.0 μg/mL, ADP 300.0 μg/mL and AMP 262.0 μg/mL, preserved in refrigerator at 4°C for later use.

Chromatographic conditions

C18 chromatographic column, 250 mm × 4.6 mm, 5 μm, column temperature 25°C, detective wavelength 254 nm, gradient elution with mobile phase 100 mmol/L potassium dihydrogenphosphate-dipotassium hydrogen phosphate buffer (pH = 6.0) [Table 1], flow-rate 0.7 mL/min, injection volume 10 μL.

Establishment of standard curve and detection of samples

To draw respectively the control mixture 1 μL, 2 μL, 3 μL, 4 μL, and 5 μL and get the standard curves on the basis of the concentration and measure peak area (mAU): ATP: y = −27.3268x + 0.5658 (R = 0.9998), ADP: y = −9.5499x + 0.5761 (R = 0.9999), AMP: y = −18.3040x + 0.2822 (R = 0.9998) (y: concentration, x: mAU). Then, the samples were detected to obtain HPLC characteristic spectrum of ATP, ADP, AMP, based on mAU of the samples and the standard curves [Figure 1], the concentration (or content) of ATP, ADP, AMP were achieved.

Detection of glucose transporter 3 mRNA in brain tissues

To take the back of right opticchiasm, total RNA was extracted by trizol method, A260/A280 of which was 1.8–2.0, purity >90%. Then RNA was transcripted reversely into cDNA with random primers, using cDNA as the template for polymerase chain reaction (PCR) amplification. GLUT3 gene: the upstream primer: 5'-GGACTTTGCTTGCTTGCTTT-3', the downstream primer: 5'-GCCCATCCTGTTTTCTTGAC-3', the amplified fragment was 377 bp; β-actin gene: the upstream primer: 5'-GAGACCTTCAACACCCCAGC-3', the downstream primer: 5'-CCACAGGATTCCATACCCAA -3', the amplified fragment was 446 bp. PCR was performed under the following conditions: Reaction system 20 μL, 95°C for 2 min, 94°C for 45 s, 60°C for 30 s and 72°C for 2 min, 35 cycles, then 72°C for 10 min. To detect the products of PCR amplification by 1% agarose gel electrophoresis, scan the target band with ChampGel5500 gel image system and menstruate the integral optical density (IOD) of the objective band with pro-plus image analysis software. The ratio of the IOD values of the objective gene band and β-actin band was taken as the relative expression of the objective gene.

Table 1: Gradient elution schedule of KH_2PO_4/K_2HPO_4

Time (min)	A (K_2HPO_4, mL)	B (KH_2PO_4, mL)
0	13.2	86.8
11.99	13.2	86.8
12.00	14.5	85.5
21.99	14.5	85.5
22.00	13.2	86.8
38.00	13.2	86.8

Detections of p-adenosine monophosphate-activated protein kinaseα1/2 and glucose transporter 3 proteins in brain tissues

To take the left brain tissues to extract the total protein and the protein concentration was determined using a modified BCA protein assay kit. The protein sample was mixed into 20 μL total volume with 1/3 volume of 4 × sodium dodecyl sulfate gel sample buffer, boiled for 5–10 min, separated for 2–3 h with 100 V electrophoresis, transferred into the poly vinylidene fluoride membrane for 1 h with 300 mA constant current. Then, the membrane was closed for 3 h with tris-buffered saline (TBS) solution, washed for 3 times with TBS Tween, incubated respectively with β-actin antibody (1:1000), p-AMPKα1/2 antibody (1:1000), GLUT3 antibody (1:500), overnighted at 4°C, washed for 3 times. Finally, the membrane was continued to incubated with HRP-coupled secondary antibody (1:1000) at room temperature for 1 h, washed for 3 times, colored using the enhanced DAB chromogenic substrate kit, scaned, placed in the image analysis system of pro-plug 6.0 to test the IOD of the objective band. Using β-actin of the total protein as an internal reference, and the ratio of the IOD values of the objective protein band and β-actin protein band as the relative expression of the target protein.

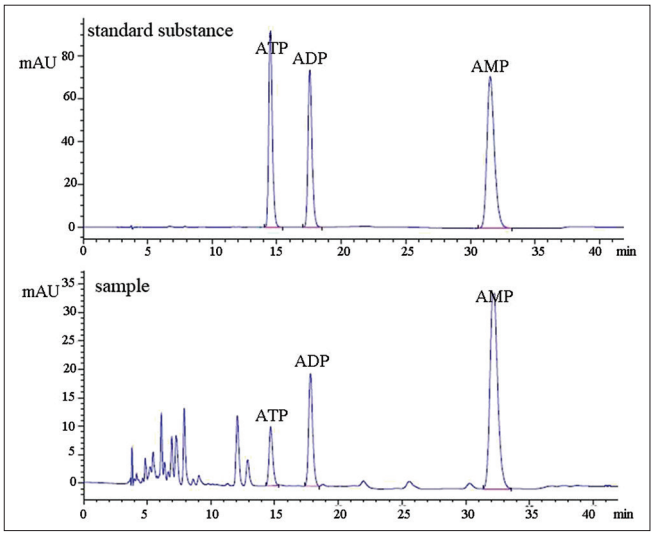

Figure 1: High performance liquid chromatography characteristic spectrum of adenosine triphosphate, adenosine diphosphate, adenosine monophosphate

Statistical analysis

Statistical Package for the Social Sciences version 16.0 (SPSS incorporated, Chicago, IL, USA) was used for all analyses. All values were expressed as a mean ± standard deviation. The mean comparison of each group was analyzed with one-way analysis of variance if the data were normally distributed and had homogeneity of variance. Log transformation was used in the analysis if the data did not accord with variance homogeneity. The homogeneity square deviation between every two groups was tested by least significant difference test. P <0.05 were considered statistically significant.

RESULTS

Comparison of the index of energy metabolism in brain tissues among the groups

Compared with the sham group, ATP, ADP, AMP contents and TAN value of brain tissues were significantly decreased in the model group (all $P < 0.01$). Compared with the model group, ATP, ADP, AMP contents, and TAN value were significantly increased in the treatment groups ($P < 0.01$ or $P < 0.05$), and the effects in AST IV + Rg1, AST IV + Rb1 were better than those in Rg1, Rb1, and AST IV alone, respectively ($P < 0.01$ or $P < 0.05$); the effects of AST IV + R1 on ATP, ADP contents and TAN value were greater than those of R1 and AST IV alone (all $P < 0.01$). In four active components combination group, the increases of ATP, ADP were more obvious than those in four active components alone, AST IV + Rg1 and AST IV + Rb1 (all $P < 0.01$), the increase of AMP was superior to that in four active components alone (all $P < 0.01$), the increase of TAN was stronger than that in four active components alone and two active components combination groups ($P < 0.01$ or $P < 0.05$). Statistically significant differences in ATP, ADP, AMP contents and TAN value were not achieved between four active components combination and edaravone (all $P > 0.05$) [Table 2].

When compared to the sham group, EC value was remarkably decreased in the model group ($P < 0.01$) while significantly increased in AST IV + Rg1, AST IV + Rb1, AST IV + R1 and four active components combination groups ($P < 0.05$ or $P < 0.01$). EC value in AST IV + Rb1, AST IV + R1 was higher than that in Rb1, R1 and AST IV alone, respectively ($P < 0.05$ or $P < 0.01$), being higher in AST IV + Rg1 than that in Rg1 ($P < 0.05$). The increase of EC value in four active components combination was stronger than that in four active components alone and AST IV + Rg1, AST IV + Rb1 ($P < 0.05$ or $P < 0.01$), presented no significant difference from that in edaravone ($P > 0.05$) [Table 2].

Comparison of p-adenosine monophosphate-activated protein kinaseα1/2 protein expression in brain tissues among the groups

Compared with the sham group, p-AMPKα1/2 protein expression was evidently up-regulated in the model group ($P < 0.05$), had further increase in AST IV, R1, four active components combination, AST IV + Rg1 and AST IV + R1 compared to the model group ($P < 0.05$ or $P < 0.01$). The increased effect on p-AMPKα 1/2 protein in AST IV + Rg1, AST IV + R1 was better than that in Rg1, R1 and AST IV alone (all $P < 0.05$); in four active components combination group, the increase of p-AMPKα1/2 protein was greater than that in four active components alone and AST IV + Rb1 (all $P < 0.05$), had no significant difference compared to edaravone ($P > 0.05$) [Figure 2].

Comparison of glucose transporter 3 mRNA expression in brain tissues among the groups

In comparison with the sham group, GLUT3 mRNA was

Table 2: Comparison of the index of energy metabolism in brain tissues among the groups ($\bar{x}$±SD, n=8)

Group	ATP (×10^{-3} μg/mL)	ADP (×10^{-3} μg/mL)	AMP (×10^{-3} μg/mL)	TAN (×10^{-3} μg/mL)	EC
Sham	101.52±9.99	250.52±11.17	426.42±25.28	778.46±21.58	0.29±0.01
Model	41.08±5.84◆◆	170.79±10.49◆◆	294.97±24.34◆◆	506.84±23.53◆◆	0.25±0.02◆◆
AST IV	63.90±6.83△△,▲▲	189.09±13.13△,▲▲	375.55±25.91△△,▲▲	628.54±23.62△△,▲▲	0.25±0.01▲▲
Rg1	58.76±5.34△△,▲▲	188.93±15.63△,▲▲	364.13±22.50△△,▲▲	611.82±29.57△△,▲▲	0.25±0.01▲▲
Rb1	58.55±7.83△△,▲▲	190.59±17.56△△,▲▲	373.11±21.91△△,▲▲	622.24±21.77△△,▲▲	0.24±0.02▲▲
R1	64.07±8.72△△,▲▲	192.97±15.61△△,▲▲	375.79±27.92△△,▲▲	632.83±23.67△△,▲▲	0.25±0.02▲▲
Four active components combination	96.56±9.43△△,**,□□,■■,☆☆	232.38±14.25△△,**,□□,■■,☆☆	413.10±20.75△△,**,□□,■■,☆☆	742.04±12.76△△,**,□□,■■,☆☆	0.29±0.02△△,**,□□,■■,☆☆
AST IV+Rg1	80.09±6.75△△,**,□□,▲▲	212.53±12.79△△,**,□□,▲▲	404.66±26.39△△,*,□□	697.28±29.85△△,**,□□,▲▲	0.27±0.01△,□,▲
AST IV+Rb1	83.52±9.96△△,**,■■,▲▲	211.92±17.59△△,**,■■,▲▲	405.56±25.10△△,*,■	701.00±38.84△△,**,■■,▲▲	0.27±0.02△△,*,■■,▲
AST IV+R1	87.43±9.72△△,**,☆☆	221.75±13.04△△,**,☆☆	399.99±22.09△△	709.17±31.33△△,**,△△,▲	0.28±0.01△△,**,☆☆
Edaravone	98.17±8.06△△	235.62±15.25△△	421.15±29.87△△	754.94±29.60△△	0.29±0.01△△

**P<0.01 versus sham; △P<0.05, △△P<0.01 versus model; *P<0.05, **P<0.01 versus AST IV; □P<0.05, □□P<0.01 versus Rg1; ■P<0.05, ■■P<0.01 versus Rb1; ☆☆P<0.01 versus R1; ▲P<0.05, ▲▲P<0.01 versus four active components combination. SD: Standard deviation; TAN: Total adenine nucleotides, AST IV: Astragaloside IV, ATP: Adenosine triphosphate, ADP: Adenosine diphosphate, AMP: Adenosine monophosphate

markedly up-regulated in the model group ($P < 0.05$), further increased in the treatment groups versus the model group ($P < 0.05$ or $P < 0.01$). The increased effect on GLUT3 mRNA in AST IV + Rg1, AST IV + Rb1, AST IV + R1 was more remarkable than that in Rg1, Rb1, R1 and AST IV alone ($P < 0.05$ or $P < 0.01$); In four active components combination group, the increase of GLUT3 mRNA was greater than that in four active components alone and AST IV + Rb1 group ($P < 0.05$ or $P < 0.01$), had no significant difference compared to edaravone ($P > 0.05$) [Figure 3].

Comparison of glucose transporter 3 protein expression in brain tissues among the groups

In comparison to the sham group, the expression of GLUT3 protein was up-regulated significantly in the model group ($P < 0.05$), compared with the model group, AST IV, Rg1, R1, four active components combination, AST IV + Rg1, AST IV + Rb1, AST IV + R1 could significantly increase GLUT3 protein expression in brain tissues ($P < 0.05$ or $P < 0.01$). The expression of GLUT3 protein in AST IV + Rg1 was higher than that in AST IV, Rg1 alone (all $P < 0.05$), being more in AST IV + Rb1 than that in Rb1 ($P < 0.05$); In four active components combination group, the increased effect on GLUT3 protein was better than that in four active components alone, AST IV + Rb1 and AST IV + R1 ($P < 0.05$ or $P < 0.01$), the difference was not statistical significance compared to edaravone ($P > 0.05$) [Figure 4].

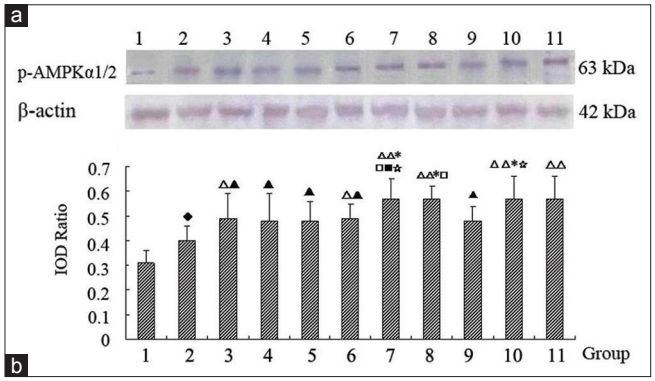

Figure 2: Protein expression of phosphorylation of adenosine monophosphate-activated protein kinaseα1/2 among the groups. (a) Western-blotting pattern of phosphorylation of adenosine monophosphate-activated protein kinaseα1/2. (b) Comparison of phosphorylation of adenosine monophosphate-activated protein kinaseα1/2 protein expression among the groups - 1: Sham, 2: Model, 3: Astragaloside IV, 4: Rg1, 5: Rb1, 6: R1, 7: Four active components combination, 8: Astragaloside IV + Rg1, 9: Astragaloside IV + Rb1, 10: Astragaloside IV + R1, 11: Edaravone. Values represent the mean ± standard deviation from the eight mice in each group; ◆$P < 0.05$ versus sham; △$P < 0.05$, △△$P < 0.01$, versus model; *$P < 0.05$, versus astragaloside IV; ▫$P < 0.05$, versus Rg1; ▪$P < 0.05$, versus Rb1; ☆$P < 0.05$, versus R1; ▲$P < 0.05$, versus four active components combination

DISCUSSION AND CONCLUSION

Brain is the most active organ during energy metabolism, oxygen consumed by brain tissues accounts for about 20% of the total oxygen consumption in resting state. Neurons consume the most of oxygen in brain tissues, and more than 99% of glucose consumed are taken from the blood, therefore, brain tissues are extremely vulnerable to ischemia or hypoxia. If the cerebral blood flow is disrupted, the energy of the brain will exhaust so quickly that brain functions are lost and the extensive brain tissues damages immediately occur. Therefore, it is very important for the treatment of cerebral ischemia to increase the content of energy and its utilization, to improve the energy metabolism in brain tissues following recovering cerebral blood flow as soon as possible.

As the main energy source of brain tissues, ATP plays an important role in maintaining physiological functions of brain cells such as active transport, protein synthesis, synaptic transmission, neurotransmitter release and intake.[9] TAN level and EC value are the important parameters that measure metabolic state of tissues and cells. TAN size reflects the oxidative respiratory activity of mitochondria and the ability of generating high-energy phosphate. Cellular energy metabolism state can be expressed by EC, a dynamic parameter reflecting the cellular energy balance which can effectively assess the status of energy reserves

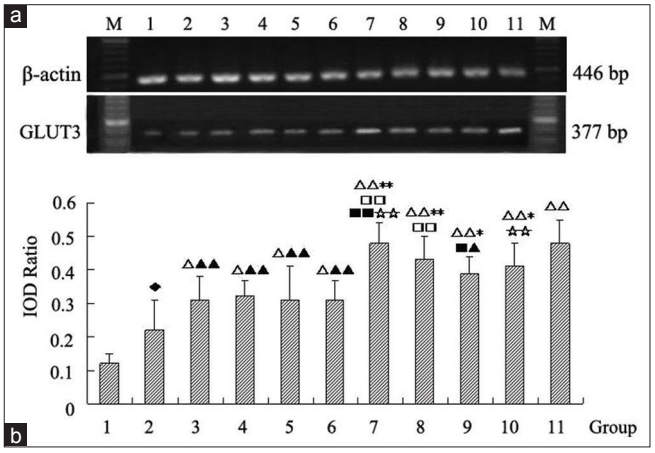

Figure 3: Expression of glucose transporter 3 mRNA among the groups. (a) Expression map of glucose transporter 3 mRNA. (b) Comparison of glucose transporter 3 mRNA expression among the groups - 1: Sham, 2: Model, 3: Astragaloside IV, 4: Rg1, 5: Rb1, 6: R1, 7: Four active components combination, 8: Astragaloside IV + Rg1, 9: Astragaloside IV + Rb1, 10: Astragaloside IV + R1, 11: Edaravone.Values represent the mean ± standard deviation from the eight mice in each group; ◆$P < 0.05$, versus sham; △$P < 0.05$, △△$P < 0.01$, versus model; *$P < 0.05$, **$P < 0.01$, versus astragaloside IV; ▫▫$P < 0.01$, versus Rg1; ▪$P < 0.05$, ▪▪$P < 0.01$, versus Rb1; ☆☆$P < 0.01$, versus R1; ▲$P < 0.05$, ▲▲$P < 0.01$, versus four active components combination

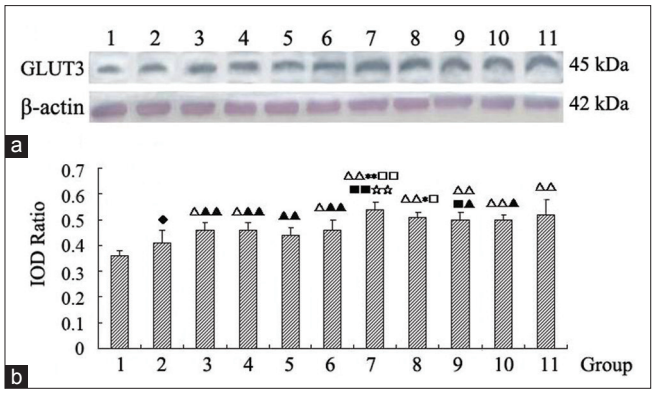

Figure 4: Expression of glucose transporter 3 protein among the groups. (a) Western-blotting pattern of glucose transporter 3. (b) Comparison of glucose transporter 3 protein expression among the groups - 1: Sham, 2: Model, 3: Astragaloside IV, 4: Rg1, 5: Rb1, 6: R1, 7: Four active components combination, 8: Astragaloside IV + Rg1, 9: Astragaloside IV + Rb1, 10: Astragaloside IV + R1, 11: Edaravone. Values represent the mean ± standad deviation from the eight mice in each group; ◆$P < 0.05$, versus sham; ▵$P < 0.05$, ▵▵$P < 0.01$, versus model; *$P < 0.05$, **$P < 0.01$, versus astragaloside IV; ▫$P < 0.05$, ▫▫$P < 0.01$, versus Rg1; ▪$P < 0.05$, ▪▪$P < 0.01$, versus Rb1; ☆☆$P < 0.01$, versus R1; ▲$P < 0.05$, ▲▲$P < 0.01$, versus four active components combination

of cells,[10] indicating active generation of ATP if EC value is high. Cerebral ischemia makes the supply of glucose and oxygen be reduced, aerobic oxidation be hindered, ATP content be decreased, ATP degradation products (ADP and AMP) be increased, and then ADP, AMP be decomposed in succession, eventually leads to the decrease of EC, TAN and the damage of brain tissues.[11]

Our results showed that AST IV, Rg1, Rb1, R1, AST IV combined with Rg1, Rb1, R1, respectively, and four active components combination could significantly increase the contents of ATP, ADP, AMP in brain tissues, raise TAN value, besides, AST IV combined respectively with Rg1, Rb1, R1 and four active components combination could markedly increase EC value. The effect of four active components combination on ATP, ADP, AMP, TAN, and EC were better than those of four active components alone or some two active components combinations. These suggested that AST IV, Rg1, Rb1, R1 had a certain improvement on energy metabolism of brain tissues after cerebral ischemia-reperfusion, AST IV combined with Rg1, Rb1, R1 could potentiate the effect, inferring that the combination of four active components could effectively promote the production of ATP or prevent its excessive consumption in ischemic brain tissues, thus relieving energy metabolism disorders, exerting neuroprotection on brain tissues.

AMPK is a serine-threonine kinase, a heterotrimer that consists of three subunits, the catalytic subunit (a subunit) has two isoforms a1 and a2, a2 subunit is distributed extensively throughout the mouse brain, being the predominant catalytic subunit.[12] AMPK can be activated by ATP depletion and the correlative stimuli for example exercise, starvation, hypoxia, ischemia, inhibitors of tricarboxylic acid cycle and so on,[13] being an adaptive response to stress process. Through adjusting the functional status or gene transcription of the enzyme related to metabolism, activated AMPK switches on ATP-producing pathways including fatty acid oxidation, glycolysis and glucose transport, switches off ATP-consuming processes such as the synthesis of fatty acid, protein and glycogen. Therefore, AMPK is called the "cellular energy regulator."[13] Whether AMPK is beneficial or detrimental in the ischemic brain has engendered considerable controversy, studies have shown that prolonged activation of AMPK can exacerbate brain injury.[14] Other studies demonstrate that AMPK has an important function to nerve cell survival.[15,16] Since neuron lacks the enzymes for glycolysis and glycogen stores, p-AMPK can promote astrocytic glycolysis to provide energy for ischemic neurons within a short period of energy deficiency,[17,18] AMPK activated by 5-aminoimidazole-4-carboxyamide ribonucleoside protects hippocampal neurons against glucose deprivation and glutamate excitotoxicity,[19] protects astrocyte from ceramide-induced apoptosis.[20] These suggest that the activation of AMPK in a certain extent is useful for improving the energy metabolism of ischemic tissues. Glucose is the major fuel for the brain, the function of GLUTs is crucial for neuronal survival. The utilization of glucose in the brain depends on GLUTs, especially GLUT3.[21] Study have reported that the expression of GLUT3 in brain tissues during cerebral ischemia is increased, being a compensatory response of brain tissues to ischemia, which promotes the utilization of glucose in brain tissues, enhances the tolerance of brain tissues to hypoxia-ischemia.[22] Cidad finds that endogenous and exogenous NO can trigger glucose intake by activating GLUT3 in pathological conditions of insufficient oxygen and glucose,[23] this effect is mediated by NO-cytochrome c oxidase-AMPK signaling pathway, indicating that GLUT3-mediated glucose intake may come from the activation of AMPK, which contributes to the protective effect.

Our results showed that p-AMPKα1/2 protein expression was up-regulated along with the increases of expressions GLTU3 gene and protein in brain tissues after cerebral ischemia-reperfusion, which was consistent with the reported research, a compensatory response of nerve cells to ischemia, making energy consumption reduced while energy production increased in brain tissues, the intake of glucose increased in cells, thus enhancing the tolerance of nerve cells to ischemia. AST IV, R1 could significantly increase p-AMPKα1/2 protein expression.

Furthermore, four active components could significantly increase the expressions of GLUT3 mRNA and protein. It suggested that four effective components could further promote the activation of AMPKα1/2 and the synthesis of GLUT3 under the activation of AMPKα1/2 and the increase of GLUT3 expression ischemia-induced, thus further promoted the intake of glucose, increased the production of high energy phosphate compounds such as ATP, improved the energy metabolism of brain tissues, playing a protective effect on ischemic brain tissues. The increased effects in four active components combination on the expressions of p-AMPKα1/2 protein, GLUT3 mRNA and protein were better than those in four active components alone or some two active components combinations, indicating that the combination of four active components had the synergism on the activation of AMPKα1/2 and the synthesis of GLUT3 on ischemic brain tissues.

In summary, AST IV, Rg1, Rb1, R1 display some protective effects on brain tissues after cerebral ischemia-reperfusion, four active components combination have the potentiation on the improving of energy metabolism, the mechanism underlying might be associated with promoting the activation of AMPKα1/2, enhancing the expression of GLUT3, thus mediating glucose into neural cells, increasing the supply and intake of glucose. Therefore, reasonable combination of the four effective components will play a synergistic role for the treatment of cerebral ischemia.

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