Glycosides of cistanche improve learning and memory in rat model of vascular dementia

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Abstract

Background: Glycosides of cistanche (GC) has been widely used as a Chinese herb. This study aimed to evaluate the effects of GC on vascular dementia (VD) and explored the mechanism.

Materials and Methods: VD model was established by the ligature of the bilateral common carotid artery in adult Wistar rats, and received daily i. p. administration of saline, GC (10mg/kg body weight /d, i.p.) or oxiracetam (450mg/kg body weight /d, i.p ) for 14 days. Cognitive performance of the rats was valued by morris water maze test. The hippocampus was dissected and subjected to proteomics and immunohistochemical analysis.

Results: GC group showed significantly lower escape latency than VD group at 4 and 5 days after operation, but showed no significant difference compared with sham-operated group and oxiracetam control group. In the hippocampus, 21 protein spots in GC group showed different expression levels compared with VD group. Compared with VD group,
15 protein spots in the GC group showed the different expression. Immunohistochemistry analysis showed that P-tau protein level was significantly higher in VD model group than sham-operated group (P < 0.05). After GC treatment, P-tau protein level in VD model rats showed significant decrease compared with VD group treated with saline (P < 0.05).

**Conclusions:** GC plays a critical role to protect hippocampal neurons in VD, by decreasing P-tau phosphorylation and increasing dihydropyrimidinase-related protein 2 (DPYSL 2) expression level. Pharmacological manipulation of GC offers a new opportunity for VD treatment.

**Keywords:** vascular dementia, glycosides of cistanche, proteomics, p-tau, dihydropyrimidinase-related protein 2

**Background**

Vascular dementia (VD) is the second leading senile dementia and is mainly caused by ischemic cerebrovascular disease. VD leads to heterogeneous cognitive impairment, and new effective therapeutic drugs that treat or prevent the progression of VD are needed [1]. However, there is still no effective Chinese herb for improving cognitive impairment induced by cerebral ischemia.

Glycosides of cistanche (GC) has been widely used as a Chinese herb with neuroprotective effects. Cistanches Herba extract enhance learning and memory of mice through promoting neuronal cell differentiation, neurite growth, and synapse formation [2]. GC can prevent apoptosis in cerebellar granule neurons and exert anti-apoptosis effect by inhibiting the activation of caspase-3 and caspase-8 [3]. Echinacoside, a major active component of Cistanches Herba, is sufficient to protect neuronal cells from rotenone injury by activating Trk signaling [4]. However, the mechanisms by which GC improves
cognitive impairment in VD are still largely unknown. Tau protein is a neuronal microtubule-associated protein that stabilizes neuronal microtubules. Phosphorylation of tau protein (P-tau) affects its capacity to interact with microtubules and impacts synaptic transmission. The accumulation of neurofibrillary tangles (NFTs) consists of P-tau protein is one of the major characteristics of Alzheimer’s disease (AD)\(^5\). It is well-known that VD and AD are the two most common dementia diseases, and they share common correlation with vascular risk factors, such as hypertension, diabetes mellitus, and hypercholesterolemia\(^6\). Therefore, P-tau may be also implicated in VD.

To provide insight into the neuroprotective effects of GC, especially on VD, in the present study we evaluated the effects of GC on learning-memory function in a rat model of VD, and performed proteomics and immunohistochemical analysis on the hippocampus of VD rats. Our results showed that GC improved learning and memory in VD rats, and changed the levels of proteins such as P-tau in VD rats.

**Materials and methods**

**Animals**

Six-month old male Wistar rats (weight 230-270 g) were provided by the experimental animal center of Hubei Province, China. All experimental animal procedures were conducted according to the Animal Care Guidelines and Local Ethical Regulations of Wuhan NO.1 Hospital, China. Ethics approval number is 00014834. Animals were housed separately in groups of 4 per cage at 25°C with a 12-hour light/12 hour dark cycle and free access to food and water. Animals\(_n=45\) were randomly assigned to four groups:
model group (n=12), sham-operated group (n=11), GC-treated group (n=12), and oxiracetam–treated control group (n=10).

**Bilateral common carotid artery ligation (2-VO)**

Under deep anesthesia with 10% chloral hydrate (Tianjin Damao Chemical Reagent Factory reagent, Tianjin, China) (350 mg/kg body weight, i. p.), bilateral common carotid arteries (CCA) were carefully separated from the surrounding tissues, and then tightly ligated with the suture. Skin was subsequently reconstructed and rats were subsequently maintained at a room temperature and returned to the home cage until resuscitation. Sham-operated control rats were subjected to the same surgical procedure without CCA ligation. VD rats were treated with GC (10mg/kg body weight /d, i.p.) or oxiracetam (450mg/kg body weight /d, i.p) immediately after operation for 14 successive days. Model group were injected with the same volume of normal saline. GC and oxiracetam were gifts from Inner Mongolia Medical University (Fig1).

**Morris Water Maze Test**

MWM test was performed according to a previously described method[7]. The testing area consisted of a circular pool filled with water (25 ± 1 °C), made opaque with milk so that the rats were unable to see underwater platform 1 cm below the water surface. The pool was divided into four quadrants (called zones I, II, III, and IV) and a platform was submerged in zone II, and visual cues were placed on the wall of the testing room. The animals were placed in the water at one of four starting quadrant points, which was varied randomly over the trials. The rats were given 2 min to find the platform and sit on it for
15 s. Rats that failed to find the location within given time were gently guided to the platform and were allowed to stay on it for 15 s. An automatic tracking system was used to record the swimming pathway, latency, swimming distance and time in each zone. All rats were sacrificed at 2 weeks after the MWM test for the subsequent analysis.

**Protein extraction**

Under deep anesthesia with 10% chloral hydrate, all rats subjected to MWM test were decapitated. The hippocampal tissues were quickly harvested on ice, and then immediately frozen in liquid nitrogen. All tissues were homogenized under liquid nitrogen using a mortar and pestle, and collected in lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM Tris). Insoluble particles were removed by centrifugation at 12,000 rpm for 20 min at 4°C. Contaminated nucleic acids in the samples were disrupted by intermittent sonic oscillation for 5 min. The supernatants were collected after centrifugation at the same condition as described above. The protein concentration in the supernatants was measured by Bradford assay, and the supernatants were store at -80°C.

**Two-dimensional Gel Electrophoresis**

The protein aliquots (120 µg) was adjusted with a rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% wt/vol DTT, 0.5% IPG buffer, and a trace of bromophenol blue) in a volume of 350 µL. Isoelectric focusing (IEF) was performed in IPG strips (pH 4-7, size 22cm) at 300 V for 12 min, 700 V for 18 min, 1500 V for 1.5 h, 9900 V for 3 h, 9990 V for 6.5 h, and then 600 V for 20 h on a Ettan IPGphor II system (GE ETTAN IPGPHOR3). After the IEF program, the strips were equilibrated in an IPG equilibration
buffer I (6 M urea, 2% SDS, 30% glycerol, 0.375M Tris, pH 8.8, 20 mg/ml DTT, and a trace of bromophenol blue), and then alkylated (25 mg/ml iodoacetamide instead of DTT in an equilibration buffer) for 15 min. 2-DE was performed in 12.5% SDS polyacrylamide gels (24 cm×19.5 cm×1.0 mm) with 0.5% agarose sealing glue, using Ettan DALT Six electrophoresis system (GE ETTAN DALTsix, PA, USA). Electrophoresis was carried out at 2 W for 45 min, followed by separation at 17 W for 4 h until the bromophenol blue nearly reached the bottom of the gels. The protein spots were visualized via silver staining (Tianjin Damao Chemical Reagent Factory) in analytical gels. 2-DE was performed in triplicate and from three independent protein extractions for each group.

**Preparative gel electrophoresis**

Protein samples (600 µg) from four groups of rats were subjected to 2-DE following the method described above. The protein spots were visualized via Coomassie brilliant blue staining (Tianjin Damao Chemical Reagent Factory) in preparative gels.

**Image Acquisition and Analysis**

The gel images were captured on a 2-DE Image Scanning system (UMAX Powerlook1100, VT, USA) and analyzed by Image Master 2D Platinum 5.0 software (GE, PA, USA). The protein spots were detected automatically and then edited manually to remove streaks, speckles, and artifacts.

**Matrix-assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry**
For peptide mass fingerprinting and subsequent analysis, gels were sliced, and Coomassie stained spots were destained and washed with 100 mM ammonium bicarbonate and acetonitrile, reduced with DTT at 60°C for 40 min, and then alkylated by IAA for 30 min in the dark. The gel was incubated in 50 μL of 12 ng/μl modified trypsin solution in 50 mM ammonium bicarbonate (pH 8.6) at 37°C overnight. Peptides were extracted from the gel plug with 1% formic acid/2% acetonitrile and concentrated using C-18 Zip-Tips. Digests were spotted (four replicates) on a MALDI target using α-cyano 4-hydroxy cinnamic acid (2 mg/ml in 50% acetonitrile, 0.1% TFA containing 10 mM ammonium phosphate) as matrix. Spectra were acquired on a 4700 MALDI TOF/TOF mass spectrometer (Applied Biosystems, NY, USA), and analyzed by GPS Explorer TM (Applied Biosystems) software.

Immunohistochemistry
Coronal hippocampal sections were fixed in 4% paraformaldehyde before being embedded in paraffin, and then cut into 4-μm thick sections. Deparaffinized coronal hippocampal sections were processed to quench endogenous peroxidase activity using 3% H₂O₂ in phosphate-buffered saline. Nonspecific immunoreactions were blocked at room temperature for 15 min. Sections were then incubated with P-tau antibody and secondary antibody at room temperature for 30 min, and were visualized following 3,3’-diaminobenzidine tetrahydrochloride (Dako, Tokyo, Japan) reaction. Images were captured using a light microscope (Olympus, Tokyo, Japan) and processed using Photoshop software (version 7.0, Adobe, San Jose, CA, USA).
Statistical Analysis

Quantitative data were expressed as means ± SD. One-way analysis of variance (ANOVA) was performed by using SPSS 10.0 analysis software. P < 0.05 was considered significant.

Results

GC promotes learning and memory in VD rats

To evaluate the effects of GC on cognitive function of VD rat model, we performed Morris water maze test. In MWM test, GC group showed significantly lower escape latency than VD group at 4 and 5 days after operation, but showed no significant difference compared with sham-operated group and oxiracetam control group (Table 1, Fig 2). These data suggest that GC promotes learning and memory in VD rats.

GC changes proteomic profiles of the hippocampus of VD rats

By proteomic analysis of rat hippocampus, we found that 21 protein spots in GC group showed different expression levels compared with VD group. Among them, four proteins showed significant difference in the hippocampus of GC rats (Table 2): including three upregulated proteins thioredoxin-like protein 1 (TXNL1), dual specificity mitogen-activated protein kinase kinase 1 (MAPKK1) and dihydropyrimidinase-related protein 2 (DPYSL2), The typical two-dimensional gel electrophoresis (2-DE) profiles of DPYSL2 were shown in Fig. 3. And one downregulated protein glutathione synthetase (GCL). Compared with VD group, 15 protein spots in the GC group showed the different
expression. And ultimately levels of 3 proteins (Tab 3), as followed: heat shock protein 75 (HSP 75) and actin-related protein 3 (ARP 3), were distinctly upregulated, and 1 protein keratin, type II cytoskeletal 6A (KRT 6A) was significantly downregulated.

**GC modulates P-tau level in the hippocampus of VD rats**

By immunohistochemistry analysis, we found that P-tau protein level was significantly higher in VD model group than sham-operated group ($P < 0.05$). After GC treatment, P-tau protein level in VD model rats showed significant decrease compared with VD group treated with saline ($P < 0.05$), but showed no significant difference compared with oxiracetam control group (Fig. 4).

**Discussion**

VD is due to the impairment of memory and cognitive functioning mainly caused by cerebrovascular diseases. The two-vessel occlusion (2VO) model is simple and stable, and recognized as the standard model of cerebral ischemia. In this study we used 2VO model to establish VD animal model. By MWM test, we found that VD group showed decreased cognitive ability compared to sham-operated group, confirming that we established VD model successfully. Based on this model, we found that GC treatment improved spatial cognitive ability of VD rats to a similar extent with oxiracetam, a positive control. These data indicate that GC could promote spatial learning and memory impairment in VD.

To investigate the mechanisms by which GC exerts neuroprotective effects on VD, we employed proteomic approach to screen differentially expressed proteins in the
hippocampus of VD rats. We found that several proteins related with energy metabolism, protein folding, signaling pathway, and cytoskeleton showed different expression in the hippocampus of VD rats.

One of the differentially expressed proteins is DPYSL2, also known as collapsin response mediator protein-2 (CRMP-2), which is enriched at the distal part of growing axons in primary hippocampal neurons and is crucial for axon differentiation and neuronal outgrowth[8,9]. Furthermore, CRMP-2 promotes microtubule assembly and mediates Ras signaling to enhance multiple-axon formation and neuronal polarity[10,11]. In this study, protein expression level of CRMP-2 was increased by 2.28 times in VD group compared to sham operation group, suggesting an important role for CRMP-2 in potential repair mechanisms of neural network formation and maintenance of neuronal polarity in VD. These data also indicate the endogenous repair of spatial cognitive ability during the process of MWM. Therefore, in our future study, we are about to investigate the learning and memory function for a long-term analysis.

Furthermore, in the present study we found higher P-tau in VD model group, but lower P-tau expression after GC treatment. Tau protein plays a key role in the morphogenesis of neurons. In certain pathological situations, P-tau protein may generate aberrant aggregates that are toxic to neurons, by affecting mitochondrial function[12]. Previous studies suggest that GC can enhance mitochondrial energy metabolism with anti-oxidation function[13,14,15]. Interestingly, CRMP-2 phosphorylation has also been characterized as a constituent of neurofibrillary tangles in Alzheimer's disease[16,17]. CRMP2 is commonly phosphorylated by cyclin-dependent protein kinase-5 (Cdk5) and
glycogen synthase kinase-3β (GSK3β) in brain of AD patients, the same kinases that phosphorylate tau protein to generate NFTs\textsuperscript{[18]}\textsuperscript{[18]}. Further studies are needed to examine whether GC regulates P-tau and CRMP2 expression level in VD by regulating Cdk5 and GSK3β activity.

**Conclusions**
In summary, our data suggests that GC may play a critical role in protection mechanism in hippocampal neurons of VD, by decreasing P-tau phosphorylation that involved neurodegeneration and in relation with CRMP2. Pharmacological manipulation of GC offers a new opportunity for the development of therapy against VD.

**Abbreviations**

- two-dimensional gel electrophoresis-based approach (2D-DIGE), Glycosides of cistanche (GC), vascular dementia (VD), morris water maze testing (MWM), Phosphorylated Tau Protein (P-tau), neurofibrillary tangles (NFTs), bilateral common carotid arteries (CCA), thioredoxin-like protein 1 (TXNL 1), dual specificity mitogen-activated protein kinase kinase 1 (MAPKK 1), Dihydropyrimidinase-related protein 2 (DPYSL 2), glutathione synthetase (GCL), collapsin response mediator protein-2 (CRMP-2), Matrix-assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF-MS), cyclin-dependent protein kinase-5 (Cdk5) and glycogen synthase kinase-3β (GSK3β)

**Competing interests**

No conflict of interest has been declared by the authors.
Authors’ contributions

SNZ and YMZ conceived this study, JC wrote the manuscript. JC and YMZ carried out the experiment and executed statistical analysis.

Acknowledgements

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References


14. Wong HS, Ko KM. Herba Cistanches stimulates cellular glutathione redox cycling by reactive oxygen species generated from mitochondrial respiration in H9c2


Fig1. The manufacture procedure and formulation of GC

Fig2. Escape latency of place navigation of various group rats.

Fig3. 2DE profile of DPYSL 2 in sham operation group and VD model group. A, The panel shows enlarged regions of the 2DE protein profile representing changes in protein amount of DPYSL 2. B, DPYSL 2 identification by peptide fingerprinting.

Fig4. GC regulate P-tau expression in the VD hippocampus. A, IHC staining in sham-operated group (a), model group (b), GC-treated group (c), and oxiracetam–treated control group (d), respectively. scale bar=22.9 um. Arrows indicate positive cells. B, Quantification of the average gray value of positive cells (P-tau), P<0.05.
Table 1  Escape latency of place navigation of various group rats

<table>
<thead>
<tr>
<th>Group</th>
<th>The first Day(s)</th>
<th>The next day</th>
<th>The third day</th>
<th>The fourth day</th>
<th>The fifth day</th>
</tr>
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<tbody>
<tr>
<td>Oxiracetam control group(n=10)</td>
<td>89.0±15.2</td>
<td>83.6±27.2</td>
<td>71.2±25.4</td>
<td>50.6±18.9 ▲△</td>
<td>45.6±15.7 ▲△</td>
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<td>VD model group(n=12)</td>
<td>82.7±22.3</td>
<td>82.2±25.1 ▲</td>
<td>71.5±31.7</td>
<td>68.3±9.2</td>
<td>60.2±18.9</td>
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<tr>
<td>GC group(n=12)</td>
<td>91.4±10.7</td>
<td>73.3±23.5</td>
<td>57.6±28.1</td>
<td>41.2±20.0△</td>
<td>38.6±15.2△</td>
</tr>
<tr>
<td>Sham operation group(n=11)</td>
<td>90.1±18.1</td>
<td>60.4±14.3</td>
<td>54.0±18.8△</td>
<td>39.7±11.1△</td>
<td>32.5±9.9△</td>
</tr>
</tbody>
</table>

▲ p<0.05 vs. Sham-operation group; △ p<0.05 vs. VD group

Table 2  4 protein spots in the VD model group showed the different expression

<table>
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<th>spot</th>
<th>Accession</th>
<th>Identified protein name</th>
<th>Function</th>
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<tr>
<td>1</td>
<td>gi</td>
<td>18266686</td>
<td>thioredoxin-like protein 1</td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>13928886</td>
<td>Dual specificity mitogen-activated protein kinase kinase 1</td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>40254595</td>
<td>Dihydropyrimidinase-related protein 2</td>
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<td>4</td>
<td>gi</td>
<td>149030882</td>
<td>glutathione synthetase</td>
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</table>
Table 3  3 protein spots in the GC group showed the different expression

<table>
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<th>Function</th>
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<td>84781723</td>
<td>heat shock protein 75 kDa HSP75</td>
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<tr>
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<td>gi</td>
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<td>actin-related protein 3 Arp3</td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>155369696</td>
<td>keratin, type II cytoskeletal 6A KRT 6A</td>
</tr>
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</table>
cistanche original medicinal materials

Reflux extraction with 2000ml, 80% ethanol (Normal atmospheric temperature), 12h/time, 4t times.

Filter

Reflux extraction liquid Steamed to 800ml

In the Resin (DM130) overnight

Filter

Centrifugation and vacuum drying

Glycosides of cistanche

Figure 1
Figure 2

The figure shows a bar chart representing the average time (in seconds) spent by subjects in different experimental groups across five days. Each column represents a different group: Oxiracetam control group, VD model group, GCs group, and Sham operation group. The bars are accompanied by error bars indicating variability. Significant differences are marked with asterisks (*), indicating statistical significance between the groups.
Figure 3

(a) sham operation group
(b) VD model group
Figure 4