Effect of glycosides of Cistanche on the expression of mitochondrial precursor protein and keratin type II cytoskeletal 6A in a rat model of vascular dementia

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Abstract  
Glycosides of Cistanche (GC) is a preparation used extensively for its neuroprotective effect against neurological diseases, but its mechanisms of action remains incompletely understood. Here, we established a bilateral common carotid artery occlusion model of vascular dementia in rats and injected the model rats with a suspension of GC (10 mg/kg/day, intraperitoneally) for 14 consecutive days. Immunohistochemistry showed that GC significantly reduced p-tau and amyloid beta (Aβ) immunoreactivity in the hippocampus of the model rats. Proteomic analysis demonstrated upregulation of mitochondrial precursor protein and downregulation of keratin type II cytoskeletal 6A after GC treatment compared with model rats that had received saline. Western blot assay confirmed these findings. Our results suggest that the neuroprotective effect of GC in vascular dementia occurs via the promotion of neuronal cytoskeleton regeneration.  

Key Words: nerve regeneration; vascular dementia; glycosides of Cistanche; mitochondrial precursor protein; keratin type II cytoskeletal 6A; proteomics; neuroprotection; neural regeneration  

Graphical Abstract  
Glycosides of Cistanche may offer a novel therapeutic agent for vascular dementia

Introduction  
Vascular dementia is the second most common type of dementia (Neltner et al., 2014; Sachdev et al., 2014; Tatlisumak et al., 2014; Sinclair et al., 2015; Thomas et al., 2015; Reijmer et al., 2016). The unremitting and irreversible memory damage that occurs in patients with vascular dementia leads to a severe deterioration in quality of life and places a heavy economic burden on the patient’s family (Barker et al., 2014; Burke et al., 2014; Chen et al., 2014; Brandenburg et al., 2016). Prevention and treatment of the disease is increasingly important in countries with aging populations. Therefore, there is increasing research interest in the search for effective drugs for the treatment of vascular dementia. Cistanche is a plant used as a nootropic in traditional Chinese medical theory (Chen et al., 2014; Szalárdy et al., 2015; You et al., 2015). Glycosides of Cistanche (GC) is a preparation extracted from Cistanche. There is considerable evidence supporting the idea that GC can improve axonal regeneration (Procaccio et al., 2014; Love et al., 2015; Brandenburg et al., 2016; Gu et al., 2016). Numerous animal
studies have shown that GC enhances cognitive function (Procaccio et al., 2014; Talarowska et al., 2014; Fischer and Maier, 2015).

We previously used bilateral common carotid artery occlusion to establish rat models of vascular dementia. In this model, spatial learning and memory are significantly improved after intervention with GC (Chen et al., 2015). GC can also regulate and improve immunity, but is best known as a nerve tonic and memory enhancer (Chen et al., 2014; Procaccio et al., 2014; Szalárdy et al., 2015; You et al., 2015; Brandenburg et al., 2016).

However, few studies have investigated the molecular mechanisms underlying the effects of GC in vascular dementia. Here, we use proteomics to explore the relationship between proteins and vascular dementia, to determine the mechanism underlying the neuroprotective effect of GC.

Materials and Methods

Animals

Six-week-old adult male specific-pathogen-free Wistar rats (n = 37), weighing 230–270 g, were provided by the Experimental Animal Center, Hubei Province, China (license number: SYXK (E) 2014-0030). The experiment followed the National Guidelines for the Care and Use of Laboratory Animals, and the Consensus Author Guidelines on Animal Ethics and Welfare produced by the International Association of Veterinary Editors. The manuscript was prepared in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Rats were housed in groups of three per cage at 25°C with a 12-hour light/dark cycle and free access to food and water. Rats (n = 37) were randomly assigned to a control group (n = 10), a vascular dementia group (n = 12), and a vascular dementia + GC group (GC-treated, n = 15).

Establishment of rat models of vascular dementia

Under deep anesthesia with 10% chloral hydrate, bilateral common carotid arteries of rats in the vascular dementia and GC-treated groups were carefully separated from the surrounding tissues, then tightly ligated using 10-0 suture thread on both ends, and the common carotid arteries were cut. The rats in the control group were subjected to the same surgical procedure except that the common carotid arteries were exposed but not ligated. The Morris water maze was performed according to previously described methods to verify whether the animal model had been successfully established (Chen et al., 2015).

Pharmacological treatment

Cistanche powder (Certificate No. Z20050216, Sinphar Tian-Li Pharmaceutical Co., Ltd., Hangzhou, China) was mixed with ultrapure water and extracted by TF-2000C Ultrasonicator (Tuofen, Shanghai, China) (power 100%, 30°C, 40 minutes). GC concentration was measured by assaying phenylethanoid glycosides. After injury, all rats in the GC-treated group received GC (10 mg/kg/day, 1 mL, intraperitoneally, once daily) for 14 consecutive days. Rats in the control and vascular dementia groups received saline (1 mL, intraperitoneally).

Immunohistochemistry

After 14 days of GC (or saline) administration, seven rats from each group were decapitated and the brains extracted. The cerebral cortices were removed and hippocampal tissue was carefully dissected out, and cut into 3-μm thick sections using freezing microtome. Sections of hippocampal tissue were fixed with 4% paraformaldehyde for 30–60 minutes, washed with PBS, and incubated in 3% H2O2 for 10 minutes followed by goat serum for 15 minutes, all at room temperature. Sections were then incubated in rabbit anti-rat phosphorylated p-tau primary antibody (1:1,000 in PBS; Bioworld Technology, Inc., TX, USA) and rabbit anti-rat amyloid beta (Aβ) primary antibody (1:1,000; Bioworld Technology, Inc.) in a wet box at 37°C for 2–3 hours, before incubation in the secondary antibody (goat anti-mouse IgG; Bioworld Technology, Inc.) for 15 minutes at room temperature. Specimens were visualized with 3,3′-diaminobenzidine tetrahydrochloride (Dako, Tokyo, Japan). Images were captured using a light microscope (Olympus, Tokyo, Japan) and processed using Photoshop (version 7.0; Adobe, San Jose, CA, USA). Immunopositive particles were brown, so brown staining in the cytoplasm was selected as positive reaction, and semi-quantification was carried out using an HMIAS-2000 image analysis system (Qianpin Co., Wuhan, China). In each specimen, five fields of view were selected at random and five positive cells were selected in each view and the average gray value was measured as a set of relative values.

Two-dimensional gel electrophoresis (2-DE)

After 14 days of GC or saline administration, eight rats from each group were anesthetized and decapitated, and the hippocampal tissues were quickly harvested and immediately frozen in liquid nitrogen. All tissues were homogenized in 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 13,000 x g for 20 minutes at 4°C. Contaminated nucleic acid was disrupted by intermittent sonic oscillation for 5 minutes. Samples were centrifuged again under the same conditions and the supernatants were collected. Protein concentration was measured using the Bradford assay (Beyotime Inc., China) and the samples were store at −80°C until use.

To investigate the protein expression profile in the hippocampus, 2-DE was performed according to the manufacturer’s instructions (GE Healthcare, Pittsburgh, PA, USA). The protein solution (120 μg/sample) was adjusted with rehydration buffer for a final volume of 350 μL. Isoelectric focusing was performed using IPG strips (pH 4–7, size 22 cm) on an Ettan IPG phor II system (all from GE Ettan IPGphor3, GE Healthcare). Afterwards, the strips were equilibrated for 15 minutes.

2-DE was performed using 12.5% sodium dodecyl sul-
phate (SDS) polyacrylamide gels (24 cm × 19.5 cm × 1.0 mm) with 0.5% agarose sealing glue in an EttaN DALTSix electrophoresis system (GE, EttaN DALTSix, GE Healthcare). Electrophoresis was carried out at 2 W for 45 minutes, followed by separation at 17 W for 4 hours until the bromophenol blue had nearly reached the bottom of 2-DE gel.

The gels were stained using silver nitrate (Damao Chemical Reagent Factory, Tianjin, China) and images were captured on a 2-DE image scanning system (VT, USA). Image Master 2D Platinum 5.0 software (GE, Inc., PA, USA) was used to scan and analyze the 2-DE image.

Protein spots were excised from the gel using an automated SpotPicker (GE, Inc.) after matching and identifying differences in protein points. Subsequently, peptide information was obtained using a 4700 MALDI TOF/TOF mass spectrometer (GE, Inc.).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

For peptide mass fingerprinting and subsequent analysis, gels were sliced and subjected to a slightly modified in-gel protocol as described in the manufacturer’s instructions. There were methanol (Fisher M/4056/17), acetonitrile (Fisher A/0626/17), trypsin (Promega V5280), trypsin resolve solution (Promega V530), ammonium bicarbonate (Sigma A6141), C-18 ZipTip (Millipore ZTC18M096), and trifluoroacetic acid (GE HealthCare).

Briefly, protein spots were destained with destaining solution (30 mM K,Fe(CN)₃·100 mM Na₂S₂O₅ = 1:1) and dehydrated with 100 mM ammonium bicarbonate and acetonitrile, reduced with trichloroethyl phosphate for 20 minutes at room temperature, and then alkylated in iodoacetic acid for 30 minutes in the dark. The gel was incubated in 50 μL of 12 ng/μL modified trypsin solution in 25 mM ammonium bicarbonate, pH 8.6, at 37°C overnight. Peptides were extracted from the gel plug with 1% formic acid/2% acetonitrile, rehydrated with 100 mm ammonium bicarbonate and acetonitrile, reduced with trichloroethyl phosphate for 20 minutes at room temperature, and then alkylated in iodoacetic acid for 30 minutes in the dark. The gel was incubated in 50 μL of 12 ng/μL modified trypsin solution in 25 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. Afterwards, samples were prepared using a Prespotted AnchorChip (PAC96) target with an alpha-cyano-4-hydroxycinnamic acid matrix for 96 sample spots and 24 calibration spots. Mass spectrum peptide information was obtained using a 4700 MALDI TOF/TOF mass spectrometer. Resulting data were analyzed using a GPS Explorer (Applied Biosystems Inc., NY, USA), which invoked a MASCOT database search (Matrix Science, London, UK) using a mouse subset of the National Center for Biotechnology Information database.

Western blot assay

The hippocampus was prepared and protein concentration was measured as described above. Protein samples were loaded onto an SDS polyacrylamide gel, electrophoresed and transferred to a polyvinylidene difluoride membrane. The membrane was washed in Tris-buffered saline and Tween 20, blocked in 10% non-fat milk and 0.05% Tween in phosphate-buffered saline. The membranes were incubated overnight at 4°C with rabbit anti-mitochondrial precursor protein polyclonal antibody (1:2,000; Proteintech Inc., Chicago, IL, USA) and rabbit anti-keratin type II cytoskeletal 6A (KRT6A) polyclonal antibody (1:1,000; Proteintech Inc.) in 10% non-fat milk. The secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit (Google Biotechnology Inc., Wuhan, Hubei Province, China) was diluted (1:2,000) in 10% non-fat milk and incubated at 25°C for 1 hour. Mouse anti-rat β-actin monoclonal antibody (1:2,000; Google Biotechnology Inc.) was used as an internal reference. A western blot scanning system (V300; EPSON, Nagano-ken, Japan), and AlphaEaseFC Adobe PhotoShop (Alpha Innotech, CA, USA) were used to determine mitochondrial precursor protein and KRT6A expression levels expressed as integrated optical density normalized to β-actin.

Statistical analysis

Quantitative data were expressed as the mean ± SD. ImageMaster 2D Platinum 5.0 software (GE, Inc.) was used to scan and analyze the 2-DE information. Two-way analysis of variance was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). P < 0.05 was considered statistically significant.

Results

Effects of GC on p-tau and Aβ immunoreactivity in the hippocampus of rat models of vascular dementia

Immunohistochemistry showed that p-tau and Aβ expression was greater after 14 days of treatment with GC than in the control group. However, expression of these proteins in the GC-treated group was significantly lower than that in the vascular dementia group (P < 0.05; Figure 1).

GC regulated expression of mitochondrial precursor protein and KRT6A in the hippocampus of rat models of vascular dementia

Expression of two proteins was significantly different in the GC-treated group than in the vascular dementia group: mitochondrial precursor protein (also known as heat shock protein (HSP) 75 kDa) was upregulated, and KRT6A was downregulated (Figures 2, 3). Protein information is listed in Table 1.

Table 1 Protein information of mitochondrial precursor protein and type II cytoskeletal 6A identified by time-of-flight mass spectrometry

| Accession | Protein name                                         | Function                                                      | T/C   |
|-----------|------------------------------------------------------|---------------------------------------------------------------|-------|
| gi|84781723 | Mitochondrial precursor, heat shock protein 75 kDa | Chaperone that expresses an ATPase activity | 2.54  |
| gi|155369696 | Keratin, type II cytoskeletal 6 | Structural support to the epithelial cell | −1.58 |
Validation of proteomic data by western blot assay
Western blot results confirmed the data obtained by proteomics: mitochondrial precursor protein expression was greater, and KRT6A expression was lower, in the GC-treated group than in the vascular dementia group (Figure 4).

Discussion
Vascular dementia is a syndrome characterized by impairments in memory, behavior and cognition, and is mainly caused by cerebrovascular diseases (Barker et al., 2014; Burke et al., 2014; Oliveira et al., 2014; Szalárdy et al., 2015). The pathology common to all types of dementia, and responsible for its progression, is neurodegeneration (Foster et al., 2014; Fischer and Maier, 2015; Kalaria et al., 2015; Chen et al., 2016; Pérez-Hernández et al., 2016). Many plant extracts have therapeutic properties. The bioactivity of these compounds against neurodegeneration is mainly due to their antioxidant and anti-amyloidogenic effects (Fischer and Maier, 2015; You et al., 2015; Pérez-Hernández et al., 2016).

Cistanche is a Chinese herb that is used as a nootropic in traditional Chinese medicine. Emerging research indicates that Cistanche is beneficial in amnesia, specifically in the inhibition of memory loss progression (Procaccio et al., 2014; You et al., 2015; Pérez-Hernández et al., 2016). There is considerable evidence that GC can promote axonal regeneration and act as a nerve growth modulator (Talarowska et al., 2014; Gu et al., 2016; Xu et al., 2016). In our previous work using the Morris water maze, we found that the escape latency of rat models of vascular dementia decreased after treatment with GC, indicating that GC improves spatial learning ability. Although GC has been used extensively for its neuroprotective effect (Chen et al., 2015), the mechanism underlying this effect remains unclear.

It is well known that Aβ peptides and p-tau are highly expressed in senile plaques and neurofibrillar tangles. Aβ and tau proteins play key roles in the morphogenesis of neurons, but in certain pathological situations, they generate aberrant aggregates that are neurotoxic (Love et al., 2015; Sadigh-Eteghad et al., 2015; Sinclair et al., 2015; Thomas et al., 2015; Wang et al., 2015; Reijmer et al., 2016). The present immunohistochemistry results indicate that p-tau and Aβ protein expression in GC-treated rat models of vascular dementia was significantly greater than that in control rats, but lower than that in untreated model rats. Therefore, the neuroprotective effect of GC may occur by reducing the toxicity of Aβ and p-tau.

Our proteomic study in rat hippocampus revealed large differences in the expression of mitochondrial precursor protein, keratin, and KRT6A. In terms of function, we speculate that the neuroprotective effect of GC may be associated with the remodeling of dendritic spine structure.

Mitochondrial precursor protein belongs to the HSP70 family (Rao et al., 2014; Gutiérrez-Aguilar and Baines, 2015; McGeer and McGeer, 2015). HSP75 is involved in molecular chaperone activation and maturation, and maintains the stability and normal function of cells (Booth et al., 2014, 2016; Tavallai et al., 2015). Mitochondrial precursor protein is thought to play an important role in the transduction of some signaling pathways (Reid et al., 2014; Booth et al., 2015a, b). Recent studies have revealed that the HSP90 family regulates the operating environment of mitochondrial protein folding (An et al., 2014; Liu and Landgraf, 2015; Roberts et al., 2015; Stary and Giffard, 2015). HSP75 is localized in mitochondria and inhibits the generation of oxygen free radicals (Radu et al., 2014; Holloway et al., 2016). Our results show that the expression of HSP75 in the GC-treated group was increased 2.54-fold, suggesting that GC plays an antioxidant role, improves cellular oxidative balance, and delays apoptosis. This finding provides interesting clues about the interactions of the mitochondrial chaperone protein with other proteins.

Another differently expressed protein was KRT6A. The keratins, also called cytokeratins, are intermediate filament proteins that create an insoluble dense meshwork through the cytoplasm, giving structural support to the epithelial cell (Haricharan et al., 2014; Rorke et al., 2015; Szymanski et al., 2015). However, cytokeratins also play an active role in various cellular survival processes (proliferation and apoptosis) (Zhu et al., 2013; Schwingshackl et al., 2015; Popov and Komianos, 2016). These proteins may undergo phosphorylation and are also part of the bridging contact between the epithelial cell and its microenvironment (Lessard et al., 2013; Gil Lorenzo et al., 2014; Zhou et al., 2014; Chakrabarti et al., 2015). CK6a is the dominant isoform in the mammary gland (Bramanti et al., 2015a, b, 2016) and is upregulated in healing wound edges of the skin, indicating that cytokeratin is involved in cellular proliferation and migration. However, we are the first to show downregulation of KRT6A in the hippocampus; KRT6A expression was decreased 1.58-fold in the GC-treated group compared with the vascular dementia group. Therefore, the neuroprotective effect of GC may be associated with inhibition of neural hyperplasia during cerebrovascular ischemia.

Together, the results of our study suggest that GC exerts its learning and memory-promoting effects in rat models of vascular dementia by reducing the expression of Aβ and p-tau, thus weakening the accumulation of these two toxic proteins and, in turn, reducing neuronal toxicity. Furthermore, GC might regulate the expression of mitochondrial precursor protein and KRT6A, which are associated with synaptic cytoskeleton morphogenesis and mitochondrial energy metabolism. Therefore, our data provide new insight into the underlying mechanisms of GCs, highlighting the possibility of GCs having multiple targets, which offers a promising novel therapeutic option for the treatment of vascular dementia.

Author contributions: YMZ and YJ conceived and designed the experiments. YMZ, WM, WW, and FW performed the experiments. YMZ and WW analyzed the data. WM provided reagents/materials/analysis tools. YMZ and FW wrote the paper. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Research ethics: The study protocol was approved by the Animal Ethics Committee of the First Hospital of Wuhan. The experimental procedure followed the National Institutes of Health Guide for the Care and Use of...
Figure 1 Effect of GCs on p-tau and Aβ immunoreactivity in the hippocampus of a rat model of vascular dementia. (A–F) Representative immunohistochemical images for p-tau and Aβ staining. Cells immunopositive for p-tau (A–C) and Aβ (D–F) in control (A, D), vascular dementia model (B, E), and GC-treated (C, F) rats. Black arrows indicate immunopositive cells, with orange-brown staining in the cytoplasm indicating p-tau and Aβ immunoactivity. (G) Average gray values of p-tau and Aβ in control rats, vascular dementia rats and GC-treated rats. p-tau and Aβ immunoreactivity was greater in the vascular dementia group than in the control group. This upregulation was significantly decreased after treatment with GC. Data are expressed as the mean ± SD. *P < 0.05, #P < 0.05 (two-way analysis of variance). Experiments were conducted in triplicate. GC: Glycosides of Cistanche; p-tau: phosphorylated tau; Aβ: amyloid beta; VD: vascular dementia.

Figure 2 Two-dimensional gel electrophoresis profiles of mitochondrial precursor protein and KRT6A expression in rat models of vascular dementia with or without GC treatment. Panels show enlarged regions of the 2DE protein profile. Areas outlined in green show differently expressed protein points. Mitochondrial precursor protein expression was 2.54-fold greater in the GC-treated group than in the model group. KRT6A expression was 1.38-fold lower than in the model group. Proteomic studies were conducted in triplicate. MP: Mitochondrial precursor protein; KRT6A: keratin type II cytoskeletal 6A.

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Figure 3 Peptide mass fingerprinting.

After destained and dehydrated, peptides were extracted and concentrated using C-18 Zip-Tips. Samples were prepared using a prespotted AnchorChip (PAC96) target. Mass spectrum peptide information was obtained using a 4700 MALDI TOF/TOF mass spectrometer. Peptide mass fingerprinting of (A) mitochondrial precursor protein; (B) KRT6A (type II cytoskeletal 6A) were listed above.

Figure 4 Western blot assay of mitochondrial precursor protein and KRT6A in the hippocampus of rat models of vascular dementia.

Data are expressed as the mean ± SD. *P < 0.05, vs. control group; #P < 0.05, vs. VD group (two-way analysis of variance). Western blot assays were conducted in triplicate. GC: Glicysides of Cistanche; VD: vascular dementia; MP: mitochondrial precursor protein; KRT6A: keratin type II cytoskeletal 6A.

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