

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

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**Abstract.** – **OBJECTIVE:** Glycosides of cistanche (GC) is extracted from Xin Jiang Cistanche, which is widely used as a Chinese herb. This study aims to evaluate the effects of GC on vascular dementia (VD).

**MATERIALS AND METHODS:** The VD model was established by the ligation of the bilateral common carotid artery in adult Wistar rats, who received daily i. p. administration of saline, GC (10 mg/kg body weight/d, i.p.) or oxiracetam (450 mg/kg body weight/d, i.p) for 14 days. Morris Water Maze test valued cognitive performance of the rats. The hippocampus was dissected and subjected to proteomics and immunohistochemical analysis.

**RESULTS:** The GC group showed significantly lower escape latency than the VD group at four and five days after surgery. They showed no significant difference when compared with sham-operated group and the oxiracetam control group. In the hippocampus, the 21 protein spots in the GC group showed different expression levels compared with the VD group. This included the four proteins that showed a significant difference: three upregulated proteins thioredoxin-like protein 1, dual specificity mitogen-activated protein kinase kinase 1 and dihydropyrimidinase-related protein 2 (CRMP-2), and one down-regulated protein glutathione synthetase. Immunohistochemistry analysis showed that P-tau protein level was significantly higher in the VD model group than the sham-operated group ( $p < 0.05$ ). After GC treatment, P-tau protein level in VD model rats showed a significant decrease compared with VD group treated with saline ( $p < 0.05$ ).

**CONCLUSIONS:** The GC plays a critical role in protecting the hippocampal neurons in the VD, by decreasing P-tau phosphorylation and increasing the CRMP-2 expression level. Pharmacological manipulation of GC offers a new opportunity for VD treatment.

*Key Words:*

Vascular dementia, Glycosides of cistanche, Proteomics, P-tau, Dihydropyrimidinase-related protein 2.

## Introduction

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<sup>1</sup>. 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 an herb with neuroprotective effects. Herba Cistanches Extract enhance learning and memory of mice by promoting neuronal cell differentiation, neurite growth, and synapse formation<sup>2</sup>. Glycosides of cistanche can prevent apoptosis in cerebellar granule neurons and exert anti-apoptosis effect by inhibiting the activation of caspase-3 and caspase-8<sup>3</sup>. Echinacoside, a major active component of Herba Cistanches, is sufficient to protect neuronal cells from rotenone injury by activating Trk signaling<sup>4</sup>. 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 and is one of the major characteristics of Alzheimer's disease (AD)<sup>5</sup>. It is well-known that VD and AD are the two most common dementia diseases. They share a common correlation with vascular risk factors, such as hypertension, diabetes mellitus, and hypercholesterolemia<sup>6</sup>. Therefore, P-tau may be also implicated in VD.

We evaluated the effects of GCs on learning-memory function in a rat model of VD, and per-

formed proteomics and immunohistochemical analysis on the hippocampus of VD rats to provide insight into the neuroprotective effects of GC. 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 (10 mg/kg body weight/d, i.p.) or oxiracetam (450 mg/kg body weight/d, i.p) immediately after surgery for 14 successive days. The model group was injected with the same volume of normal saline.

### Morris Water Maze Test

The MWM test was performed according to a previously described method<sup>7</sup>. The testing area consisted of a circular pool filled with water ( $25 \pm 1$  °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 (labeled zones I, II, III, and IV) and a platform was submerged in zone II. Visual cues

were placed on the wall of the testing room. The animals were placed in the water at one of the four starting quadrant points, which was varied randomly throughout 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 a 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 two 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 22 cm) 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 on 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 four 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 wasper formed 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 (MALDI-TOF-MS)**

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 ten mM ammonium phosphate) as matrix. Spectra were ac-

quired 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% H<sub>2</sub>O<sub>2</sub> 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. Two-way analysis of variance (ANOVA) was performed by using SPSS 10.0 analysis software (SPSS Inc., Chicago, IL, USA). *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 the MWM test. In the MWM test, the GC group showed significantly lower escape latency than the VD group at 4 and 5 days after surgery, but showed no significant difference compared with the sham-operated group and the oxiracetam control group (Table I). These data suggest that GC promotes learning and memory in VD rats.

**Table I.** Escape latency of place navigation in various group rats.

| Group                    | The first day (s) | The next day | The third day            | The fourth day            | The fifth day             |
|--------------------------|-------------------|--------------|--------------------------|---------------------------|---------------------------|
| Oxiracetam control group | 89.0 ± 15.2       | 83.6 ± 27.2  | 71.2 ± 25.4              | 50.6 ± 18.9* <sup>#</sup> | 45.6 ± 15.7* <sup>#</sup> |
| VD model group           | 82.7 ± 22.3       | 82.2 ± 25.1* | 71.5 ± 31.7              | 68.3 ± 9.2                | 60.2 ± 18.9               |
| GCs group                | 91.4 ± 10.7       | 73.3 ± 23.5  | 57.6 ± 28.1              | 41.2 ± 20.0 <sup>#</sup>  | 38.6 ± 15.2 <sup>#</sup>  |
| Sham operation group     | 90.1 ± 18.1       | 60.4 ± 14.3  | 54.0 ± 18.8 <sup>#</sup> | 39.7 ± 11.1 <sup>#</sup>  | 32.5 ± 9.9 <sup>#</sup>   |

\**p* < 0.05 vs. Sham-operation group; <sup>#</sup>*p* < 0.05 vs. VD group.

### GC Changes Proteomic Profiles of the Hippocampus of VD Rats

By proteomic analysis of the rat hippocampus, we found that 21 protein spots in the GC group showed different expression levels compared with the VD group. Among them, four proteins showed significant difference in the hippocampus of the GC rats (Table II): including three upregulated proteins thioredoxin-like protein 1 (TXNL 1), dual specificity mitogen-activated protein kinase kinase 1 (MAPKK 1) and dihydropyrimidinase-related protein 2 (DPYSL2); and one down-regulated protein glutathione synthetase (GCL). The typical two-dimensional gel electrophoresis (2-DE) profiles of DPYSL2 were shown in Figure 1.

### 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 the VD model group than the sham-operated group ( $p < 0.05$ ). After the GC treatment, P-tau protein level in VD model rats showed significant decrease compared with the VD group treated with saline ( $p < 0.05$ ), but showed no significant difference compared with the oxiracetam control group (Figure 2).

## Discussion

VD is due to the impairment of memory and cognitive functioning mainly caused by cere-

brovascular diseases<sup>8,9,10,11</sup>. 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 the 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.

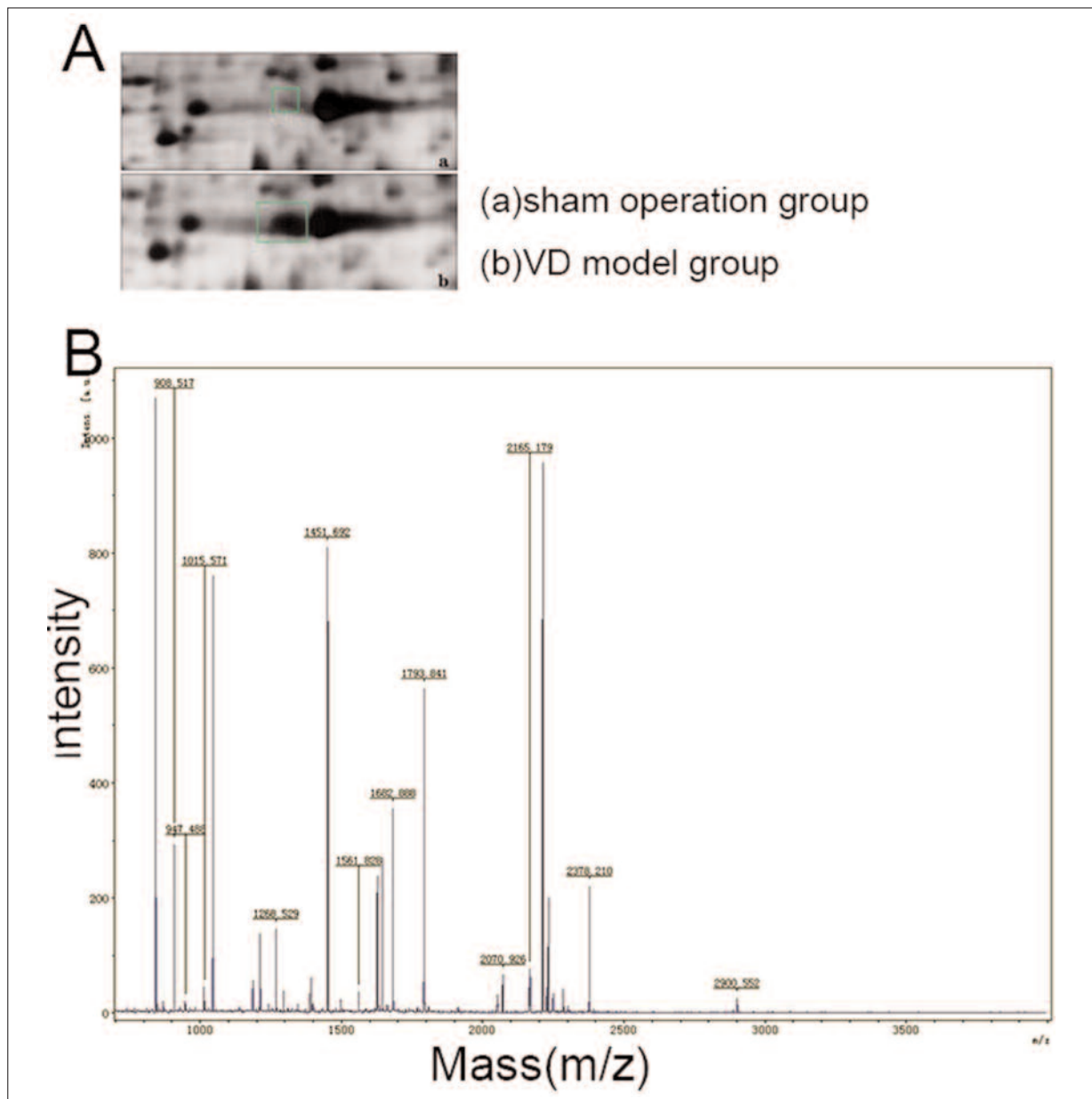
To investigate the mechanism by which GC exerts neuroprotective effects on VD, we employed proteomic approach to screening differentially expressed proteins in the hippocampus of VD rats. We found that several proteins related to energy metabolism, protein folding, signaling pathway, and cytoskeleton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

One of the differentially expressed proteins is DPYSL2, also known as collapsin response mediator protein-2 (CRMP-2), which is enriched in the distal part of growing axons in primary hippocampal neurons and is crucial for axon differentiation and neuronal outgrowth<sup>12,13</sup>. Furthermore, CRMP-2 promotes microtubule assembly and mediates Ras signaling to enhance multi-

**Table II.** Four proteins with different expression between GC and VD groups.

| Spot | Accession    | Identified protein name                                    | Function                                                                                                                                                                                                                                                                               |
|------|--------------|------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1    | gil18266686  | Thioredoxin-like protein 1                                 | Active thioredoxin with a redox potential of about -250 mV                                                                                                                                                                                                                             |
| 2    | gil13928886  | Dual specificity mitogen-activated protein kinase kinase 1 | Dual specificity protein kinase which acts as an essential component of the MAP kinase signal transduction pathway. Binding of extracellular ligand s such as growth factors, cytokines and hormones to their cell-surface receptors activates RAS and this initiates RAF1 activation. |
| 3    | gil40254595  | Dihydropyrimidinase-related protein 2                      | Plays a role in neuronal development and polarity, as well as in axon growth and guidance, neuronal growth cone collapse and cell migration. Necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton                                              |
| 4    | gil149030882 | Glutathione synthetase                                     | ATP binding glutathione binding glutathione synthase activity glutathione synthase activity magnesium ion binding                                                                                                                                                                      |



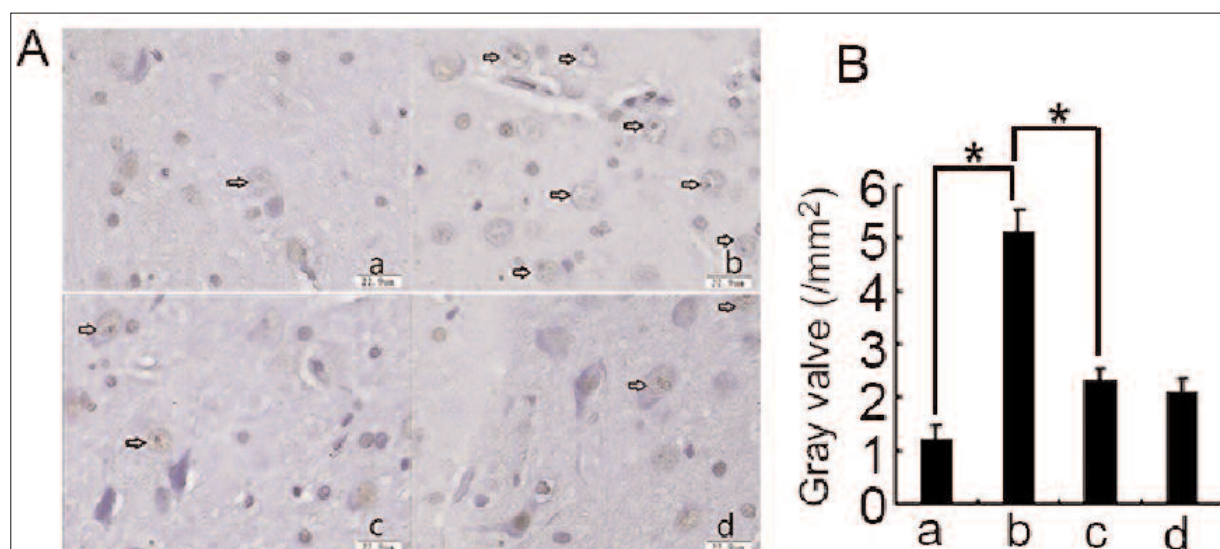


**Figure 1.** 2DE profile of DPYSL 2 in sham operation group and VD model group. **A**, The panel shows enlarged regions of the 2DE protein profile representing changes in protein amount of DPYSL 2. **B**, DPYSL 2 identification by peptide fingerprinting.

ple-axon formation and neuronal polarity<sup>14,15</sup>. In this study, protein expression level of CRMP-2 was increased by 2.28 times in GC group compared to the VD group, suggesting an important role for CRMP-2 in potential repair mechanisms of neural network formation and maintenance of neuronal polarity in VD.

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<sup>15</sup>. Previous studies suggest that GC can enhance mitochondrial energy metabolism with anti-oxidation function<sup>16,17,18</sup>. Interestingly, CRMP-2 phosphorylation has also been characterized as a constituent of neurofibrillary tangles in AD.



**Figure 2.** 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  $\mu$ m. Arrows indicate positive cells. **B**, Quantification of the average gray value of positive cells (P-tau),  $p < 0.05$ .

## Conclusions

CRMP2 is commonly phosphorylated by cyclin-dependent protein kinase-5 (Cdk5) and glycogen synthase kinase-3 (GSK3) in the brain of the AD patients, the same kinases that phosphorylate tau protein to generate NFTs<sup>19,20</sup>. Further studies are needed to examine whether GC regulates P-tau and CRMP2 expression level in VD by regulating Cdk5 and GSK3 activity<sup>21,22,23</sup>. In summary, our data suggest that GC plays a critical role to protect hippocampal neurons of VD, by decreasing P-tau phosphorylation and increasing CRMP-2 expression level. Pharmacological manipulation of GC offers a new opportunity for the development of therapy against VD.

## Acknowledgements

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## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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