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Treating Alzheimer's disease with *Yizhijiannao* granules by regulating expression of multiple proteins in temporal lobe

Hong Zhu[1], Liuyang Luo[2], Sihang Hu[1], Keli Dong[1], Guangcheng Li[1], Ting Zhang[1]

1 Department of Traditional Chinese Medicine, Third Xiangya Hospital of Central South University, Changsha, Hunan Province, China
2 Department of Emergency, Bao-an District Hospital of Traditional Chinese Medicine, Shenzhen, Guangdong Province, China

Corresponding author:
Guangcheng Li, Ph.D., Department of Traditional Chinese Medicine, Third Xiangya Hospital of Central South University, Changsha 410013, Hunan Province, China, yellingu@163.com.

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Abstract

Yizhijiannao granules have been shown to improve cognitive function in Alzheimer's disease patients. The present study sought to explore the mechanisms involved in the cognitive enhancing effects of *Yizhijiannao* granule. Senescence-accelerated mouse prone 8 mice with learning and memory disorders were intragastrically treated with *Yizhijiannao* granule for 8 weeks. Mice intragastrically treated with double distilled water for 8 weeks were considered as the control group. 2D gel electrophoresis was used to isolate total protein from the temporal lobe of senescence-accelerated mouse prone 8 mice, and differential protein spots were obtained by mass spectrometry. Thirty-seven differential protein spots were found in the temporal lobe area of both groups. Ten protein spots were identified: high mobility group box 1, dimethylarginine dimethylaminohydrolase-1, neuroglobin, hemoglobin beta adult major chain, peroxiredoxin-6, cofilin-1, flotillin 1, peptidylprolyl isomerase A, voltage-dependent anion channel-2 and chaperonin containing TCP1, and subunit 2. Among other functions, these proteins are separately involved in the regulation of amyloid beta production, oxidative stress, neuroinflammation, regulation of tau phosphorylation, and regulation of neuronal apoptosis. Our results revealed that *Yizhijiannao* granule can regulate the expression of various proteins in the temporal lobe of senescence-accelerated mouse prone 8 mice, and may be therapeutically beneficial for the treatment of Alzheimer's disease.

Key Words: *nerve regeneration; traditional Chinese medicine; neurodegeneration; Alzheimer's disease; Yizhijiannao granule; mass spectrometry; cognition; neural regeneration*

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Introduction

Alzheimer's disease is a complex disease that is associated with many dysfunctional processes (Huang and Mucke, 2012; Reiman, 2014). Modern medicines that target specific dysfunctional processes will have limited effect in the treatment of Alzheimer's disease. However, therapeutic methods and drugs that have broad-spectrum applications will provide greater outcomes (Huang and Mucke, 2012; Reiman, 2014). Traditional Chinese medicines, which are natural drugs with multiple components and functions, may be possible candidates for the treatment of Alzheimer's disease.

More and more evidence has shown that the temporal lobe plays an important role in Alzheimer's disease (Scheff and Price, 1993; Frisoni et al., 2010; Oosterman et al., 2012). Postmortem ultrastructural examination of the temporal lobe in Alzheimer's disease patients has revealed significant synapse loss. In addition, atrophy of medial temporal structures has been considered to be a valid diagnostic marker at the mild cognitive impairment stage. Temporal lobe atrophy was closely related to lower executive function, general cognitive function, and episodic memory performance in Alzheimer's disease.

The senescence accelerate mouse prone 8 is a typical senile mouse model with learning and memory impairments (Wang et al., 2014). The brains of the senescence-accelerated mouse prone 8 have some neuropathologic characteristics such as hyperphosphorylation of tau, and the overproduction of amyloid precursor protein and amyloid-beta protein, which are similar to those seen in Alzheimer's disease. Other characteristics of Alzheimer's disease shared by senescence-accelerated mouse prone 8 mice include increased oxidative damage, decreased choline acetyltransferase activity, and increased alpha synuclein. With respect to the behavioral and histopathological signatures of Alzheimer's disease, senescence-accelerated mouse prone 8 mice are currently considered to be an ideal model of Alzheimer's disease (Morley et al., 2012).

Yizhijiannao granule is a cipher prescription that has been

used for treating senile dementia for more than 13 years in our hospital. Previous studies have shown that *Yizhijiannao* granule can enhance cognitive performance in Alzheimer's disease patients and Alzheimer's disease-model mice (Yang et al., 2005; Yang and Dong, 2013). Further studies revealed that *Yizhijiannao* granule may exert its therapeutic effect by inhibiting neural cell apoptosis, reducing tau phosphorylation and relieving neuroinflammation (Yang et al., 2006; Wang et al., 2009). In addition, *Yizhijiannao* granule can inhibit early beta-amyloid (25–35)-induced PC12 cell apoptosis (Zhang et al., 2012) . Taken together, these studies suggest that the beneficial effect of *Yizhijiannao* granule involves multiple targets and pathways. Therefore, we aimed to identify target-proteins of *Yizhijiannao* granule that were particularly related to the treatment of Alzheimer's disease.

In the present study, senescence-accelerated mouse prone 8 mice were administered *Yizhijiannao* granule, and differential protein expression in the temporal lobe was identified to elucidate the multi-targeted effects of *Yizhijiannao* granule in the treatment of Alzheimer's disease.

Materials and Methods

Animals

Twenty 6-month-old male senescence-accelerated mouse prone 8 mice were obtained from the Experimental Animal Center, First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine (Tianjin, China; license No. 0003740). Mice were housed in separate cages under conditions free of specific pathogens at 21 ± 3°C with a relative humidity of 55–58%, exposed to a daily 12-hour light/dark cycle. Mice were given free access to food and water. This study was approved by the Animal Ethics Committee of Central South University, China.

Yizhijiannao granule preparation

Yizhijiannao granule was composed of seven commonly used herbs: Herba Epimedii, Herba Cynomorii, Radix Notoginseng, Radix Acanthopanacis Senticosi, Radix Dipsaci, Semen Platycladi, Hirudo. These herbs were mixed in the ratio of 3:3:2:2:2:2:1 (dry weight). The raw herbs for *Yizhijiannao* granule were purchased from the Dispensary of traditional Chinese medicine, Xiangya Third Hospital of Central South University, China. All of the herbs are regionally famous drugs and were authenticated by the herbalists of Xiangya Third Hospital. *Yizhijiannao* granule was extracted as previously described by Hangzhou Dekang Pharmaceutical Co., Ltd (Hangzhou, Zhejiang Province, China) (Zhang et al., 2012). *Yizhijiannao* granule was dissolved in distilled water for administration to mice. The dose of administration used in this study was based on our previous study (Yang et al., 2006; Wang et al., 2009).

Experimental groups

A total of 20 adult male senescence-accelerated mouse prone 8 mice were equally and randomly divided into two groups: (1) treatment group, intragastrically administrated *Yizhijiannao* granule 1 mL/d for 8 weeks; (2) control group: intragastrically administrated the same volume of double distilled water for 8 weeks. No death or infection occurred in both groups. All 20 mice were included in the final analysis without deletion.

Sample collection of brain tissue

Eight weeks following treatment, all mice were decapitated. Brain tissues were quickly segregated from the temporal lobe at near-freezing temperature, and each tissue was stored in Eppendorf tubes at −80°C in liquid nitrogen.

Total protein extraction and concentration determination

Each tissue (50 mg in average wet weight) was homogenized with 400 μL lysis solution, comprising 2 mmol/L thiourea, 7 mmol/L Urea, 100 mmol/L dithiothreitol, 5 mmol/L phenylmethyl sulfonylfluoride, 4% (v/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5 mmol/L ethylenediamine tetraacetic acid, 40 mmol/L Tris, 2% (v/v) NP-40, 1% (v/v) Triton X-100, and 2% pharmalyte). After incubation at 37°C for 1 hour, the homogenate was then centrifuged at 12,000 r/min for 1 hour to obtain the supernatant. The protein concentration of the supernatant was determined using the 2D Quant Kit (Amersham Bioscience, Buckinghamshire, UK.

2D gel electrophoresis

2D gel electrophoresis was performed according to a previous method (Gorg et al., 1995), in a horizontal electrophoresis system, IPGphor™ for the first-dimensional isoelectric focusing on IPG strips (24 cm long, linear gradient 3–10). In the second dimension of 2D-polyacrylamide gel electrophoresis, proteins with similar isoelectric points were further separated according to their molecular weight on a sodium dodecyl sulfate polyacrylamide gel.

Staining and computer analysis of the 2D gel

According to the previous blue silver staining method (Gorg et al., 2000), the gels were stained using Coomassie blue-G250. The Coomassie blue-stained gels were then scanned with an Image scanner (Bio-Rad, Hercules, CA, USA) and analyzed by LabScan software to get the gel image. The gel image was then analyzed qualitatively and quantitatively using PD Quest software (Bio-Rad). Only spots showing a difference in the integrated intensity larger than two-fold between the control group and the treated group were further studied.

Mass spectrometric analysis

The protein spots of interest were cut off the 2D gels and enzymatically degraded with modified trypsin in accordance with a previous method (Hubbard, 2006). The spots were then analyzed using the MALDI-TOF-MS (Applied Biosystems, Carlsbad, CA, USA) to get the peptide mass fingerprint. The peptide mass fingerprint data were submitted to the MASCOT software (http://www. Matrix-science.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF) search engine to identify the proteins.

Table 1 Differential protein spots identified in mass spectrometry protein sequence database

Spot No.	Database ID	Protein name	Matched peptide segment	Isoelectric point	Molecular weight (Da)	Sequence covering rate (%)	Expression
1	gi|6754208	High mobility group box 1	5/12	5.62	25,048	39	Down-regulation
2	DDAH1-MOUSE	Dimethylarginine dimethylaminohydrolase -1	13/47	5.64	31,629	55	Down-regulation
3	gi|4760594	Neuroglobin	7/31	7.97	15,851	56	Down-regulation
4	Q9CRZ2-MOUSE	Hemoglobin beta adult major chain	6/19	8.09	14,442	53	Up-regulation
5	gi|3219774	Peroxiredoxin-6	8/37	5.71	24,969	37	Up-regulation
6	COF1-MOUSE	Cofilin-1	10/29	8.26	18,645	70	Up-regulation
7	gi|123270828	Flotillin 1	11/28	6.58	48,372	63	Down-regulation
8	gi|6679439	Peptidylprollyl isomerase A	12/30	7.74	18,131	53	Up-regulation
9	gi|6755965	Voltage-dependent anion channel 2	13/30	7.44	32,340	46	Down-regulation
10	gi|5453603	Chaperonin containing TCP1, subunit 2	15/42	6.01	57,794	40	Up-regulation

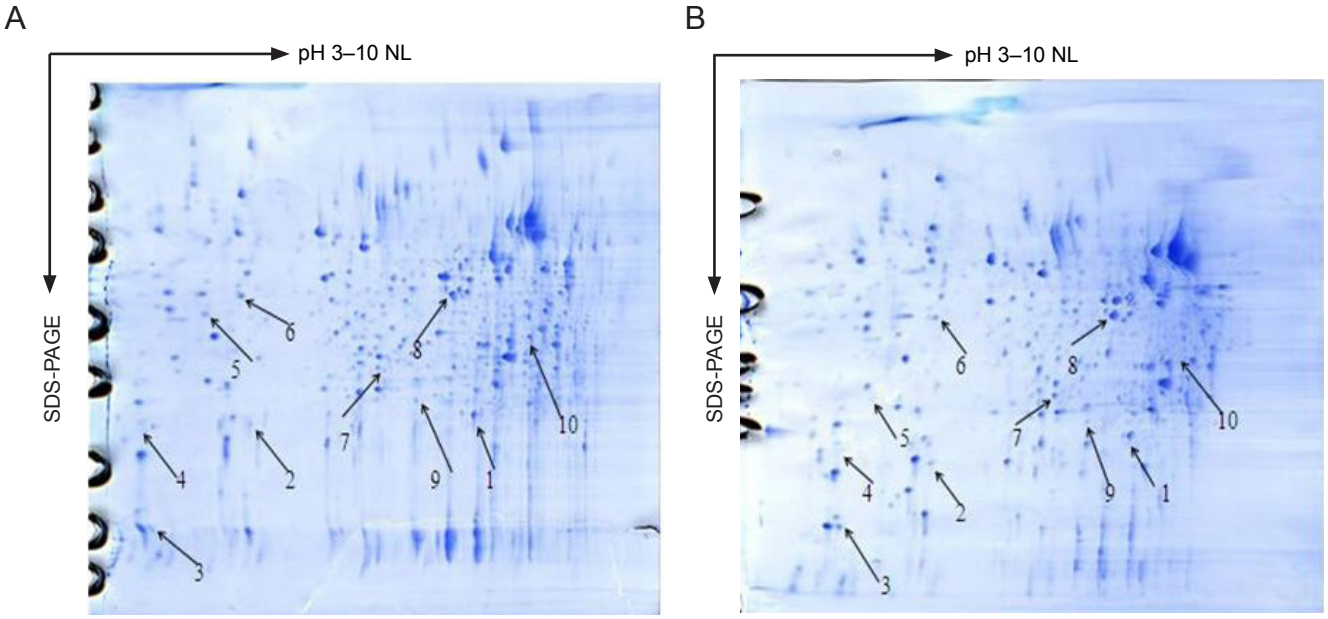

Figure 1 Two-dimensional gel electrophoresis maps in senescence-accelerated mouse prone 8 from the treatment group and the control group.
Maps from the (A) treatment group and (B) control group. Arrows indicate evident protein spots, and the figure represents the number of differential protein spots. NL: Non-liner; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis. 1–10: Differential protein spots 1–10.

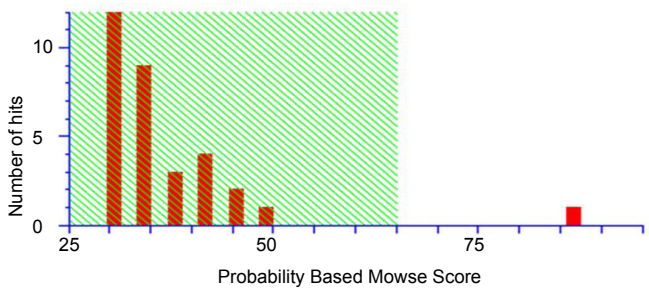

Figure 2 Database search results of No. 10 protein spot.
X-axis represents Mowse scores, and scores larger than those in the shadow area were considered statistically significant ($P < 0.05$). Mowse scores > 65 represent statistical significance. Y-axis represents the number of proteins with certain scores.

Results

Temporal 2D gel electrophoresis maps and differential protein spot identification

To ensure maximal reproducibility of the 2D gel electrophoresis experiment, we compared three samples from the treatment group and three samples from the control group in the studied brain regions. We obtained three 2D gel electrophoresis maps in each group. Approximately 900 spots per gel were detected using the PD Quest software. For the mass spectrometric analysis of the spots on the 2D gel electrophoresis maps, a threshold minimum of a two-fold up- or down-regulation in the integrated intensity between the control group and the treatment group was used to exclude proteins that differed in integrated intensity owing to small variations

occurring randomly during the experimental process. Finally, 10 differentially expressed protein spots were identified, of which 5 protein spots were up-regulated and 5 protein spots were down-regulated (**Figure 1**). The database search result of protein No. 10 is shown in **Figure 2**. The differentially expressed protein spots identified are listed in **Table 1**.

Discussion

Although the first presentation of Alzheimer's disease dates back to 1906, the pathogenic mechanisms of the disease remain inadequately understood and fragmentary, some of which are in a state of serious controversy. Thus, we tend to deem that Alzheimer's disease is a complex disease consisting of multipathogenic mechanisms that have multifactorial involvement. In our present study, the identified proteins could be assigned to several categories related to amyloid beta production, oxidative stress, neuroinflammation, cell apoptosis, and tau phosphorylation.

One of the pathologic hallmarks of Alzheimer's disease is the extracellular aberrant accumulation of amyloid beta in senile plaques. Recently, it was shown that lipid rafts played an important role in the amyloidogenic processing of the amyloid precursor protein; lipid rafts could function as platforms for amyloid beta production (Vetrivel and Thinakaran, 2010). The identified protein flotillin-1 has been used as a biochemical marker of lipid rafts in neural tissue (Girardot et al., 2003; Kokubo et al., 2003), and its expression is increased in the brains of Alzheimer's disease subjects and increases in parallel to increasing stages of amyloid beta deposition (Kania et al., 2012). In our study, flotillin-1 was down-regulated in the treatment group, thus affording fewer platforms for amyloid beta production. Therefore, it is tempting to speculate that *Yizhijiannao* granule may derive therapeutic effect by decreasing amyloid beta production in a platform-dismantling mechanism, which may be a new direction for the treatment of Alzheimer's disease. However, further research is needed.

Numerous studies on Alzheimer's disease during the past decade have consistently confirmed the involvement of oxidative stress in disease pathogenesis (Padurariu et al., 2013; Kosenko et al., 2014). Recently, more and more evidence shows that neuroglobin may act as an intracellular reactive oxidative species and a reactive nitrogen species scavenger to play a role in neuron protection (Jin et al., 2008; Li et al., 2008). Being a reactive oxidative species scavenger, it could attenuate amyloid beta neurotoxicity *in vitro*, the transgenic Alzheimer phenotype *in vivo*, and protect PC12 cells against amyloid beta-induced cell injury (Khan et al., 2007; Liu and Chan, 2014). Taken together, these evidences indicate that increasing neuroglobin expression may have a therapeutic effect in Alzheimer's disease. With neuroglobin up-regulated, our research shows that *Yizhijiannao* granule may use the antioxidant properties of neuroglobin to obtain a therapeutic effect.

There is a consensus that chronic inflammatory processes are involved in the pathogenesis of Alzheimer's disease (Liu and Chan, 2014; Serpente et al., 2014). When glial cells are activated, they release proinflammatory factors to initiate the neuroinflammatory process. High mobility group box 1, a mediator of acute and chronic inflammatory diseases (Asavarut et al., 2013), was previously reported to be significantly upregulated in Alzheimer's disease brains and colocalize with senile plaques (Takata et al., 2003). Based on the above research, we may assume that high mobility group box 1 probably plays a role in the chronic inflammatory process in Alzheimer's disease brains as a proinflammatory factor. In our study, *Yizhijiannao* granule may down-regulate high mobility group box 1 to accomplish an anti-inflammatory effect.

Another major pathologic characteristic of Alzheimer's disease is the accumulation of intra-cellular neurofibrillary tangles, which contains paired helical filaments composed of the microtubule-associated protein tau. Peptidyl-prolyl-cis-trans isomerase A (PPIase A, pin1) was shown to be involved in tauopathies in Alzheimer's disease, and the levels of soluble pin1 are decreased in the brains of Alzheimer's disease patients (Lu et al., 1999a, b). Pin1 can bind to phosphorylated tau at Thr-231, dephosphorylating the phosphorylated tau and facilitating conformational changes (Kimura et al., 2013), thereby restoring its biological function of binding microtubules and enhancing microtubule assembly. The decrease of pin1 in brains of Alzheimer's disease patients may break the balanced phosphorylation state of tau proteins, leading to tau hyperphosphorylation and neurofibrillary tangle formation. In view of the evidence presented above, we presume that pin1 could protect against tau hyperphosphorylation and thus inhibit the formation of neurofibrillary tangles. In our study, pin1 was up-regulated in the treated group, so *Yizhijiannao* granule may play a part in the regulation of tau phosphorylation to produce a therapeutic effect.

Voltage-dependent anion channel is considered to be a global regulator, or governor, of mitochondrial function (Lemasters and Holmuhamedov, 2006; Colombini, 2012), participating in many cellular processes, especially in cell apoptosis (Shoshan-Barmatz et al., 2010). Voltage-dependent anion channel-2, up-regulated in our study, was reported to be capable of inhibiting BAK activation and mitochondrial apoptosis, with cells deficient in voltage-dependent anion channel-2 exhibiting enhanced BAK oligomerization and being more susceptible to apoptotic death (Cheng et al., 2003). Conversely, overexpression of voltage-dependent anion channel-2 selectively prevented BAK activation and inhibited the mitochondrial apoptotic pathway (Cheng et al., 2003). Therefore, voltage-dependent anion channel-2 may facilitate a neuroprotective function in Alzheimer's disease based on its anti-apoptotic property. By up-regulating voltage-dependent anion channel-2, *Yizhijiannao* granule may be involved in this anti-apoptotic process.

Proteomics has been considered to be a useful tool to analyze protein changes in complex diseases such as Alzheimer's disease, but there are also some shortcomings for this analytical tool that should be taken into consideration; the main problem is that proteomics cannot isolate all the low-abundance proteins, some of which may own strong bioactivities and play important roles in Alzheimer's disease onset and

treatment. Therefore, further research is needed to verify the results of proteomics studies. Collectively, in the present study, we have identified some proteins which could be potential therapeutic targets for Alzheimer's disease treatment. *Yizhijiannao* granule could induce a remedial effect on Alzheimer's disease owing to its targeting of multiple pathways, which include reducing amyloid beta production, anti-oxidant, anti-inflammatory and anti-apoptotic properties, and dephosphorylation of tau proteins.

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Conflicts of interest: *None declared.*

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