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

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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 Notopterygii, 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 Chinese medicine, Xiangya Third Hospital of Central South University, China. The gel image was then analyzed qualitatively and quantitatively 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 twofold 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        | Q6CRZ2- 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|123720828         | Flotillin 1                           | 11/28                  | 6.58                  | 48,372                    | 63                         | Down-regulation |
| 8        | gi|6679439           | Peptidylprolyl 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.

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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 Tinakaran, 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., 2010). Being a reactive oxidative species scavenger, it could be assigned to several categories related to 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.

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**Table 1**

| Protein Name | Description |
|--------------|-------------|
| Pin1         | 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 oligimerization 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. |
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.

**Yizhijiangao** 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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**Author contributions:** ZHU designed this study, analyzed data and wrote the draft of the manuscript. LIOO and HU SH were responsible for data integration. LIGC authorized the manuscript and guided the study. DONG KL provided technical suggestions. ZHU T was in charge of animal preparations. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

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