Shen-Zhi-Ling oral solution improves learning and memory ability in Alzheimer’s disease mouse model

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Abstract

OBJECTIVE: To investigate the effector mechanisms and effector targets of Shen-Zhi-Ling (SZL) oral solution in the treatment of Alzheimer’s disease (AD).

METHODS: In this study, we carried out gavage with SZL oral solution in an APP/PS-1 heterozygous double transgenic AD mouse model for 12 continuous weeks. Haematoxylin and eosin staining, Nissl staining and Annexin V/Propidium Iodide staining were used to detect the brain histopathology in AD mouse model. Immunofluorescence staining was used to detect the expression levels of autophagy’s proteins. Morris water maze test was used to detect the learning and memory ability in AD mouse model.

RESULTS: Pathological results showed that neuronal loss in the hippocampus of mice in the SZL intervention group was significantly alleviated and the number of apoptotic neurons was significantly decreased compared with the control group (physiological saline and non-intervention groups). Immunofluorescence staining results showed that the expression of autophagy activators, Beclin-1 and LC3B, was significantly increased in the hippocampal neurons of mice of the SZL intervention group, while the expression of the apoptotic factor, caspase-3, was significantly decreased. At the same time, hippocampal accumulation of Aβ42 protein was significantly decreased. In addition, results of the water maze experiment showed that the latency period in mice from the SZL intervention group was significantly reduced.

CONCLUSION: In summary, we believe that the SZL oral solution significantly activates autophagy in hippocampal neurons, effectively reducing the accumulation of Aβ42 peptides, alleviating neuronal injury and apoptosis, and ultimately improving the cognitive function in a mouse model of AD.

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Keywords: Alzheimer disease; Autophagy; Shen-Zhi-Ling oral solution

INTRODUCTION

Autophagy refers to the detachment of sections of bilayer membranes from the ribosome-free attachment region in rough endoplasmic reticulum, which envelops...
some cytoplasm, and organelles, proteins, etc., in the cell that requires degradation to form autophagosomes. These autophagosomes fuse with lysosomes to form autophagolysosomes capable of degrading its contents in order to achieve the cell's metabolic requirements and renewal of some organelles. Autophagy in a cell will undergo a series of steps: formation of phagosomes -> formation of autophagosomes -> formation of autophagolysosomes -> digestion of internal contents. During this process, the formation of autophagosomes is an important step, and the diameter of autophagosomes is generally around 300–900 nm, with an average of 500 nm. Common contents of autophagosomes include cytoplasmic components and some organelles, such as mitochondria, endosomes, peroxisomes, etc. Compared with other organelles, the half-life of autophagosomes is very short (around 8 min), showing that autophagy is an effective response to environmental changes. The effector substrates of autophagolysosomes are endogenous, and are derived from senescent or disintegrating organelles, or local cytoplasm inside the cells. Autophagolysosomes are surrounded by a single-layer membrane, and their contents often contain undegraded endoplasmic reticulum, mitochondria, and Golgi bodies, or lipids, and glycogen. In normal cells, autophagolysosomes play important roles in digestion, degradation, natural replacement of intracellular structures. When a cell is under the effects of drugs, radiation, or mechanical injury, the number of autophagolysosomes significantly increase. Autophagolysosomes are also often found in diseased cells. Various types of macromolecules inside phagolysosomes are degraded to simpler products under the action of hydrolases. Examples of this process include protein degradation into dipeptides or free amino acids, nucleic acid degradation into nucleosides and phosphates, carbohydrates degradation into oligosaccharides or monosaccharides, neutral lipids degradation into glycerol and fatty acids. These soluble small molecules that are produced by degradation can enter the cytoplasmic matrix through lysosomal membranes and be reused in cellular metabolism. Some undigested substances remain in the phagolysosomes and form residual bodies. Many studies have pointed out that disorders in neuronal autophagy occur during neurodegenerative diseases. At the same time, autophagy levels in neurons significantly decrease during the development of Alzheimer's disease. First, researchers found that both AD patients and AD animal models show varying degrees of defects in autophagy in brain tissues. In the neuronal dendrites of 8-week-old APP/PS1 double transgenic mice, the number of autophagic vesicles is 5 times higher than that in WT mice of the same age, and number further increases to 23-fold in 9-month old mice (APP/PS1 vs WT). At the same time, the aggregation of many immature autophagic vesicles is observed in brain tissues of AD patients. Recent studies have pointed out that there is a widespread loss of Beclin-1 in neurons in AD pathophysiology. In senescent neurons, Beclin-1 expression is generally decreased, suggesting impaired autophagy. However, caspase-3 activity will significantly increase in brain tissues during AD development, which results in depletion of neuronal Beclin-1. Researchers constructed a transgenic mouse with a Beclin-1 (F121A) mutation to induce AD. Results showed that autophagic activity was generally higher in neurons and other tissues in these mice and can significantly reduce the deposition of Aβ protein. The researchers speculated that Beclin-1 mutation results in the disruption of the binding between Beclin-1 and its inhibitory factor, Bcl2. In addition, researchers also found that PTEN-induced PINK1 loss is associated with AD pathology. PINK1-deficient mAPP mice exhibit increased cerebral Aβ42 accumulation, mitochondrial abnormalities, learning and memory impairment, and synaptic plasticity at an earlier age than mAPP mice. On the other hand, PINK1 overexpression can activate autophagy receptors, OPTN and NDP52, and thereby increase autophagic signalling, promote the clearance of damaged mitochondria in neurons, and alleviate synaptic loss and cognitive decline in Aβ42-induced AD mice. Study results showed that rescuing defective autophagy functions could alleviate the course of AD. Therefore, much evidence suggests that autophagy may be a potential target for AD treatment.

Currently, the US FDA has approved 5 drugs for the treatment of AD, which are donepezil, memantine, galantamine, rivastigmine, and tacrine. However, clinical data showed that these drugs can only alleviate mild AD symptoms and not slow down the progression of dementia. Therefore, it is necessary to develop alternative drugs for AD. Many studies have showed the Chinese herbal medicine is accepted and used by patients, and that these medicines have fewer side effects. Therefore, this is a promising class of prospective alternative medicines. The Shen-Zhi-Ling (SZL) oral solution is the first Traditional Chinese Medicine (TCM) formula that has been approved in China for treating patients with mild to moderate AD. Clinical and experimental studies have shown that SZL can improve the cognitive function of mild to moderate AD patients. However, the effector mechanisms and effector targets of SZL oral solution are currently unknown and require in-depth research and exploration.

In this study, our hypothesis is that the SZL oral solution promotes autophagic activity in hippocampal neurons and can alleviate neuronal injury and improve cognitive function in an AD mouse model.

**MATERIALS AND METHODS**

**Grouping and drug intervention of APP/PS1 transgenic mice**

A total of 30 APPswe/PS1 ΔE9 (APP/PS1) heterozy-
gous double transgenic mice were used. Mice were six-month-old males with a weight of (26 ± 3) g. In addition, 10 wild-type six-month-old C57BL/6 male mice with a weight of (27 ± 2) g were also used in this study. The APP/PS1 transgenic mice all express human/mouse chimeric amyloid precursor protein and human mutant presenilin-1 protein. SZL is a TCM compound oral solution that is made from 10 types of TCM components, containing 74. 5 g of raw medicine per 10 mL of oral solution. These components are Danshen (Radix Salviae Miltiorrhizae), Guizhi (Ramulus Cinnamomi), Baishao (Radix Paeoniae Alba), Gancao (Radix Glycyrrhizae), Fuling (Poria), Ganjiang (Rhizoma Zingiberis), Yuanzhi (Rhizoma Paeonii), Shichangpu (Rhizoma Acori Tatarinowii), Longgu (Os Draconis), Muli (Concha Ostreae). Detailed extraction and quality control information was obtained from the manufacturer (Shandong Wohua Pharmaceuticals Co. , Ltd. , Weifang, China). APP/PS1 mice were divided into three groups of 10 mice each, namely AD model group, SZL intervention group, and negative control group. The AD model group (APP/PS1 group) contained APP/PS1 mice that did not undergo treatment. SZL intervention group (APP/PS1 + SLZ group) consisted of APP/PS1 transgenic mice. SZL oral solution (2.5 g/mL) was administered to these mice by gavage (200 μL/20 g) every day for 12 continuous weeks. The negative control group (APP/PS1 + NS group) consists of APP/PS1 transgenic mice. Physiological saline was administered to these mice by gavage (200 μL/20 g) every day for 12 continuous weeks. In addition, ten C57BL/6 mice that were not treated with any drug were used a wild-type control group (WT). The aforementioned mice were housed in an identical environment (22 ± 3 °C, 60% relative humidity, 12-h light/12-h dark cycle) and ad libitum food and water were provided. All procedures were performed in accordance with the Guide for the Care and Use of Medical Laboratory Animals and the guidelines of the Shanghai University of Traditional Chinese Medicine (Shanghai, China) Laboratory Animal Care and Use Committee.

**Immunofluorescence staining**

Mouse brain tissue sections were deparaffinized and rehydrated. Sections in citrate buffer were placed in a boiling water bath for 10 min for antigen retrieval. The sections were then placed at room temperature to gradually cool to room temperature before the boiling procedure was repeated again. Following that, the sections were left to cool to room temperature. Sections were blocked at room temperature for two hours using 5% BSA. The corresponding primary antibody (Table 1) was diluted at a ratio of 1:50 before blocking for one hour and incubation overnight at 4 °C with shaking. Following that, the sections were washed with PBS for 15 min before the sections were incubated with secondary antibody for 1 h at room temperature, and diluted in a 1:500 ratio with the blocking solution. The sections were washed with PBS for 45 min before the addition of antifade mounting medium containing DAPI. A fluorescent microscope was used for observation and imaging of the sections using 488 and 555 nm lasers.

**HE staining**

Mouse brain tissues were fixed in 10% formaldehyde before routine dehydration and paraffin embedding. Continuous sections (5 μm) were made from the brain. Following that, tissue dewaxing, haematoxylin and eosin (HE) staining, ethanol elution, xylene clearing, and mounting with neutral resin were carried out. A light microscope was used to observe the pathological morphology of the sections.

**Nissl staining**

Nissl staining was carried out according to the manufacturer’s instructions for the Nissl Staining Kit (Beyotime Biotechnology, Zhejiang, China). Mouse brain tissue was sectioned, deparaffinized and rehydrated. Nissl staining solution was used to stain sections for 10 min. The sections were washed twice with distilled water, for 10 min each time, before dehydration with 95% ethanol for 5 s. Xylene was used to clear the tissue before neutral resin was used for mounting. A light microscope was used to observe the pathological morphology of the sections.

**Annexin V/Propidium Iodide (PI) staining**

Annexin V/PI staining was performed according to the manufacturer’s instructions for the Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology, Zhejiang, China). In brief, mouse brain tissue was sectioned, deparaffinized and rehydrated. A staining solution containing Annexin V-FITC conjugate solution (195 μL), Annexin V-FITC reaction solution (5 μL), and propidium iodide (10 μL) was combined in a centrifuge tube and gently mixed. Subsequently, the section was incubated with the solution, in the dark at room temperature for 20 min. The stained sections were observed under a fluorescence microscope. Annexin V-FITC appears as green fluorescence while PI appears as red fluorescence.

**Morris water maze (MWM) test**

The protocol by Xing et al. was used as a reference. The maze (diameter of 120 cm) was filled with water and the hidden platform was submerged one cm below the water surface. The temperature of the water was maintained at 20-23°C. In a five-day experiment, every mouse was trained to locate the hidden platform. The interval between experiments was 30-40 min and this was carried out for 5 d. Mice were first left on the platform for 30 s. Mice were allowed to explore the platform for 70 s, before being placed in the water again. The time taken for the mouse to find the platform was recorded as the escape latency. On day 6, the platform was removed before mice were placed in the maze for 70 s of exploration. During this period, the time spent...
in every quadrant and across the platform and the total path length were recorded. During the experiment, a video camera was connected to the recorder and computer tracking system.

RESULTS

Effectiveness of SZL oral solution on hippocampal neurons

From the HE and Nissl staining of hippocampal sections in normal, elderly mice, we can see that hippocampal neurons were arranged neatly, neurons were intact, synapses were thin and long, cell body was full, nuclei were deeply stained, and cell status was good (Figure 1). In the APP+/PS1+ double transgenic AD mouse model, hippocampal neurons exhibited significant atrophy and apoptosis, cell body staining was light and blurry, synapses and cell nuclei were few, and cell status was significantly poor (Figure 1). In the SZL oral solution intervention group, the hippocampi showed several neurons with intact morphology, and a full cell body; deep staining; intact, thin, and long synapse, and nuclei were observed with the deep blue stain (Figure 1). The histopathological staining results of the physiological saline group were consistent with the APP+/PS1+ double transgenic AD mouse model (Figure 1).

SZL oral solution can effectively rescue hippocampal neurons from autophagy

First, immunofluorescence staining results showed that, with the exception of mice from the normal group, positive expression of Aβ42 peptide was observed in APP+/PS1+ double transgenic AD mice, mice from the SZL intervention group and the physiological saline group (Figures 3, 7). However, the positive expression levels of the Aβ42 peptide in the hippocampi of APP+/PS1+ double transgenic AD mice showed significant green and red fluorescence, with a distribution area that was significantly greater than the normal group. Similarly, the mice from the SZL oral solution intervention group showed green and red fluorescence. However, distribution of the green and red fluorescent areas was significantly smaller than in the hippocampi of mice in the model group. The histopathological staining results of the physiological saline group were consistent with the APP+/PS1+ double transgenic AD mouse model (Figure 2).

Figure 1 Histopathological staining of mouse hippocampal tissues
A-H: hematoxylin-eosin staining shows that the SZL oral solution can effectively alleviate apoptosis in hippocampal neurons (A-D: ×100; E-H: ×200). I-P: Nissl staining shows that the SZL oral solution can effectively alleviate apoptosis in hippocampal neurons (I-L: ×100; M-P: ×200). A, E, I, M: WT; B, F, J, N: APP+/PS1+ (APP+/PS1+ transgenic mice); C, J, K, O: APP+/PS1+SZL (APP+/PS1+ transgenic mice treated with SZL oral solution); D, H, L, P: APP+/PS1+NS (APP+/PS1+ transgenic mice treated with normal saline). APP: amyloid beta precursor protein; PS1: presenilin 1; WT: wild type; SZL: Shen-Zhi-Ling; NS: normal saline.
The distribution areas of green and red fluorescence in the hippocampi of mice from the SZL oral solution intervention group were significantly decreased. A: WT; B: APP+/PS1+ (APP+/PS1+ transgenic mice); C: APP+/PS1+SZL (APP+/PS1+ transgenic mice treated with SZL oral solution); D: APP+/PS1+NS (APP+/PS1+ transgenic mice treated with normal saline); E: this shows that apoptosis and necrosis of hippocampal neurons was significantly ameliorated. PI: Propidium Iodide; APP: amyloid beta precursor protein; WT: wild type; PS1: presenilin 1; SZL: Shen-Zhi-Ling; NS: normal saline. P < 0.01 vs WT group; P < 0.01 vs APP+/PS1+ group; n = 10.

Figure 3 Immunofluorescence staining of Aβ42 peptides
A, E, I, M: Aβ1-42; B, F, J, N: Tuj1; C, G, K, O: DAPI; D, H, L, P: Merge. A-D: WT; E-H: APP+/PS1+ (APP+/PS1+ transgenic mice); I-L: APP+/PS1+SZL (APP+/PS1+ transgenic mice treated with SZL oral solution); M-P: APP+/PS1+NS (APP+/PS1+ transgenic mice treated with normal saline). The expression of Aβ42 peptides in the hippocampi of mice from the SZL oral solution intervention group was significantly lower than the model and physiological saline groups. Aβ1-42: Amyloid-β1-42; Tuj1: Neuronal Class III β-Tubulin; DAPI: 4,6-diamidino-2-phenylindole; WT: wild type; APP: amyloid beta precursor protein; PS1: presenilin 1; SZL: Shen-Zhi-Ling; NS: normal saline.
the physiological saline group (Figures 4, 7). However, the positive expression levels of caspase-3 in the hippocampi of mice from the SZL oral solution intervention group was significantly lower than the model and physiological saline groups (Figures 4, 7).

Third, immunofluorescence was used to measure Beclin-1 and LC3B protein expression during autophagy. The results showed the hippocampi of normal mice showed high Beclin-1 and LC3B protein expression (Figures 5-7). However, the levels of Beclin-1 and LC3B proteins in the APP+/PS1+ double transgenic AD mice, and mice from the SZL intervention group and the physiological saline groups were all significantly decreased (Figures 5-7). Importantly, the expression of Beclin-1 and LC3B proteins in the hippocampi of mice from the SZL oral solution intervention group was significantly higher than the model and physiological saline groups.

**SZL oral solution can effectively improve learning and memory impairment in AD mice**

The experiment results showed that there were no significant differences in the time taken to find the platform in the target quadrant (latency) and the total path length between the APP+/PS1+ double transgenic AD mice, and mice from the SZL intervention group and the physiological saline groups (Figure 8). When MWM tests were conducted after three months of drug intervention, the experimental results showed that latency in mice from the SZL intervention group was significantly decreased when compared with APP+/PS1+ double transgenic AD model mice and mice from the physiological saline group (Figure 8). The experimental results showed that there was a significant difference in latency between the APP+/PS1+ double transgenic AD mice and mice from the physiological saline intervention group (Figure 8). In addition, the total path length taken by the SZL treated mice was significantly shorter compared with the APP+/PS1+ double transgenic AD model mice and mice from the physiological saline intervention group (Figure 8). There was no significant difference in the total path length between the APP+/PS1+ double transgenic AD mice and mice from the physiological saline group (Figure 8).
In this study, we hypothesize that Chinese herbal medicine is beneficial in delaying the onset and progression of these diseases, and in neuronal protection. Many studies have pointed out that AD patients showed good compliance and less side effects when taking Chinese herbal medicine for a long period of time. This drug can significantly ameliorate AD-associated pathological symptoms. Of the several components in SZL, dang shen can enhance intelligence; dried Polygala tenuifolia roots, Acorus tatarinowii and China root can improve cognitive function; dragon’s bone and Concha Ostreae are widely known to have sedative effects and various studies showed that they can induce sleep and is therefore suitable for treating sleep disorders. Lastly, Chinese peony and honey fried with liquorice root can effectively improve cerebral circulation. Therefore, the composition of the SZL oral solution has nerve-soothing effects and can promote cerebral circulation and improve the brain microenvironment. SZL oral solution has some efficacy in clinical practice.

We considered that cellular autophagy is an essential physiological function to maintain cell vitality. We selected the commonly used AD (APP+/PS1+) double transgenic model and SZL oral solution was administered as an intervention. The results of the study showed that AD mice that were given SZL oral solution showed significant improvements in hippocampal neuron phenotypes. In the model group, hippocampal neurons exhibited apoptosis and necrosis (caspase 3 was significantly overexpressed and Annexin V/Propidium Iodide staining was positive). However, after intervention with SZL oral solution, the hippocampus showed several neurons with good status. We also analysed apoptosis-related biomarkers and found that the degree of apoptosis in hippocampal neurons in AD mice from the SZL intervention group was significantly lower than in the model and physiological saline groups. APP: amyloid beta precursor protein; PS1: presenilin 1; WT: wild type; SZL: Shen-Zhi-Ling; NS: normal saline.
Figure 6 Immunofluorescence staining of the autophagy protein, LC3B

A, E, I, M: LC3B; B, F, J, TuJ1; C, G, K, O: DAPI; D, H, L, P: Merge. A-D: WT; E-H: APP+/PS1+ (APP+/PS1+ transgenenic mice); I-L: APP+/PS1+–SZL (APP+/PS1+ transgenenic mice treated with SZL oral solution); M-P: APP+/PS1+–NS (APP+/PS1+ transgenenic mice treated with normal saline). The expression of LC3B protein in the hippocampi of mice from the SZL oral solution intervention group was significantly higher than in the model and physiological saline groups. LC3B: Microtubule-Associated Protein 1 Light Chain 3 Beta; TuJ1: Neuronal Class III β-Tubulin; APP: amyloid beta precursor protein; DAPI: 4',6-diamidino-2-phenylindole; PS1: presenilin 1; WT: wild type; SZL: Shen-Zhi-Ling; NS: normal saline.

Figure 7 Quantitative statistical of immunofluorescence staining results

(a) Aβ1-42. (b) Caspase 3. (c) Beclin. (d) LC3B. Aβ1-42: Amyloid-β1-42; LC3B: microtubule-associated protein 1 light chain 3 Beta. \(^{a}P < 0.01\) vs APP+/PS1+ group; \(n = 10\).
increased expression. At the same time, their $\alpha$B42 levels were significantly decreased. These findings suggest that SZL oral solution can increase the ability of neurons in $\alpha$B42 clearance through autophagy activation. This process maintains normal physiological and biochemical activity in cells, ultimately delaying neuronal apoptosis. Finally, we employed the Morris water maze to test the learning and memory of AD mice from various groups. The results also showed that the memory of AD mice from the SZL intervention group were significantly superior to mice from the non-intervention group.

The main pharmacological basis of SZL oral solution is kidney tonifying and essence filling. Some studies have reported that SZL oral solution can effectively alleviate mild cognitive impairment in model animals or humans. However, the above Chinese medicine alleviated and treated dementia whether is via modulating neuronal autophagy? The question has not been studied and reported. The significance is, that we revealed the new mechanism of SZL oral solution pharmacology, which indicated a new way of thinking and direction for optimizing and improving the therapeutic effect of this medicine; and that, our research also proved that the pharmacological effect of Traditional Chinese Medicine is a scientific fact of multi-target and multi-function (originally considered the main pharmacological basis of SZL oral solution was kidney tonifying).
ing and essence filling). Of course, our research still has some limitations. Our study of neuronal damage in the course of AD remains on the phenomenon of apoptosis. We should know that brain tissue is a complex organ, and the cytotoxicity caused by Aβ42 peptide is also varied. A systematic and comprehensive study of the neuronal state during the occurrence of AD will be our next research goal. In addition, our research on neuron autophagy in AD mice still has a lot of extensibility. In the future, we will combine electron microscopy technology with molecular biology assay to display autophagic phenotypes (such as autophages, autophagic lysosomes, etc.) at the microscopic and submicroscopic levels of organelles as far as possible. At the same time, proteomic methods (such as iTRAQ) systematically and comprehensively demonstrate the interaction between proteins in the process of neuronal autophagy imbalance. These existing research results and future ideas will provide a strong guarantee for the treatment of AD.

In conclusion, our findings suggest that SZL oral solution promotes autophagy in neurons, and increases Aβ 42 clearance, and delays neuronal apoptosis. This ultimately improves the learning and memory ability in the AD mouse model.

REFERENCES


