Protective effects of Bushen Tiansui decoction on hippocampal synapses in a rat model of Alzheimer’s disease

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Graphical Abstract

Abstract

Bushen Tiansui decoction is composed of six traditional Chinese medicines: Herba Epimedii, Radix Polygoni multiflori, Plastrum testudinis, Fossilia Ossis Mastodi, Radix Polygalae, and Rhizoma Acorus tatarinowii. Because Bushen Tiansui decoction is effective against amyloid beta (Aβ) toxicity, we hypothesized that it would reduce hippocampal synaptic damage and improve cognitive function in Alzheimer’s disease. To test this hypothesis, we used a previously established animal model of Alzheimer’s disease, that is, microinjection of aggregated Aβ₂₅₋₃₅ into the bilateral brain ventricles of Sprague-Dawley rats. We found that long-term (28 days) oral administration of Bushen Tiansui decoction (0.563, 1.688, and 3.375 g/mL; 4 mL/day) prevented synaptic loss in the hippocampus and increased the expression levels of synaptic proteins, including postsynaptic density protein 95, the N-methyl-D-aspartate receptor 2B subunit, and Shank1. These results suggested that Bushen Tiansui decoction can protect synapses by maintaining the expression of these synaptic proteins.

Bushen Tiansui decoction also ameliorated measures reflecting spatial learning and memory deficits that were observed in the Morris water maze (i.e., increased the number of platform crossings and the amount of time spent in the target quadrant and decreased escape latency) following intraventricular injections of aggregated Aβ₂₅₋₃₅ compared with those measures in untreated Aβ₂₅₋₃₅-injected rats. Overall, these results provided evidence that further studies on the prevention and treatment of dementia with this traditional Chinese medicine are warranted.

Key Words: nerve regeneration; neurodegeneration; Bushen Tiansui decoction; Alzheimer’s disease; synaptic plasticity; amyloid β; synaptic proteins; Shank1; N-methyl-D-aspartate receptor 2B subunit; postsynaptic density protein 95; Morris water maze; neural regeneration

Introduction

Alzheimer’s disease (AD), the major cause of dementia among the elderly, is a neurodegenerative disease characterized by memory impairment, progressive cognitive function decline, and personality and behavioral changes. More than 25 million people worldwide have been diagnosed with AD, and that figure is expected to multiply in the next few decades (Wang et al., 2015). However, no effective treatments are available to prevent the onset or progression of AD.

The hallmark neuropathological features of AD are neu-
rofibillary tangles composed of hyperphosphorylated tau protein, senile plaques composed of amyloid beta (Aβ) peptide, and the loss of synapses (Blennow et al., 2006; Gao et al., 2016). Synaptic proteins, including but not limited to Shank1, the N-methyl-D-aspartate receptor 2B (NR2B) subunit, and postsynaptic density 95 (PSD-95), are integrated at the postsynaptic density in dendritic spines (Miletic et al., 2010). Both PSD-95 and Shank1 are synaptic scaffolding proteins and play critical roles in regulating the strength of synaptic activity and dendritic spine formation (Hung et al., 2008; Tu et al., 2014). They are thought to be correlated with levels of Aβ oligomers in patients with AD and in the brains of amyloid precursor protein transgenic mice (Sultana et al., 2010; Venigalla et al., 2015). The NR2B subunit is indispensable to synaptic transmission, synaptic plasticity, and neural development, and it plays a significant role among the N-methyl-D-aspartic acid receptor subunits (Kiraly et al., 2011). Upregulation of NR2B expression enhances long-term potentiation in the hippocampal CA1 subregion in slices obtained from transgenic mice, facilitates synaptic transmission, and improves memory (Wang et al., 2009; Plattner et al., 2014). Pathological changes of these proteins may directly or indirectly affect dendritic spine and synaptic functions. Therefore, targeting these synaptic proteins may provide a therapeutic potential for reducing Aβ-induced synaptic injury and cognitive impairment.

Bushen Tiansui decoction (BTD) is a traditional Chinese medicine derived and modified from Kongshe Zhen-zhong-dan, which was included in the publication Qianjin Fang by the pharmacologist Simiao Sun. BTD is composed of six traditional Chinese medicines: Herba epimedii, Radix Polygoni multiflori, Plastrum testudinis, Fossilia Ossis Mastodi, Radix Polygalae, and Rhizoma Acorus tatarinowii. Herba Epimedii is made from the dried aerial parts of Epimedium brevicornum Maxim (family Berberidaceae). Radix Polygoni multiflori is the root tuber of the perennial vine Polygonum multiflorum Thunb (family Knotweed). Plastrum testudinis is the back shell and plastron of the animal Chinemys reeveii (family Testudinidae). Fossilia Ossis Mastodi is the ossature fossil of an ancient mammal. Radix Polygalae is the dry root of Polygala (family Polygalaceae). Rhizoma Acorus tatarinowii is the dried rhizomes of the plant Acorus tatarinowii (family Araceae).

Traditional Chinese medicine asserts that the basic pathogenesis underlying AD includes kidney essence and brain marrow deficiencies and that the fundamental treatment is to administer Bushen Tiansui. In BTD, Herba Epimedii and Radix Polygoni multiflori are thought to be the main effective ingredients. They nourish the kidney and replenish the blood and essence. We and others previously reported that icariin extracted from Herba Epimedii inhibits Aβ-induced cytotoxicity in SH-SY5Y cells by decreasing the production of peroxide hydrogen and in cortical neurons by modulating cocaine-regulated transcripts (Sha et al., 2009; Liu et al., 2015). A molecule isolated from Radix Polygoni multiflori promotes PC12 cell differentiation, increases intracellular calcium levels in hippocampal neurons, and facilitates high frequency stimulation-induced hippocampal long-term potentiation (Wang et al., 2011). The remaining active ingredients in BTD improve and play critical roles in learning, connecting the heart and kidney, and activating the nine orifices. For example, Plastrum Testudinis increases viability and reduces apoptosis in PC12 cells (Liu et al., 2011); Radix Polygalae induces autophagy via the mammalian target of rapamycin signaling pathway in PC12 cells (Wu et al., 2013); and Rhizoma Acorus tatarinowii serves as a preventive and regenerative therapeutic agent to promote neurogenesis in neurodegenerative disorders by activating extracellular signal-regulated kinase in aberrant neural progenitor cells (Mao et al., 2015). Therefore, we hypothesized that the myriad functions of BTD would antagonize Aβ neurotoxicity and inhibit neurodegenerative process and diseases, including AD.

The goals of this study were to investigate whether BTD improved memory deficits in AD by maintaining the expression of synaptic proteins and to provide evidence for the need of further studies on the prevention and treatment of dementia by this traditional Chinese medicine.

Materials and Methods

Animals

Male Sprague-Dawley rats (certificate No. 43004700010946; license No. SCXK [Xiang] 2014-0012), weighing 250–300 g, were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, Hunan Province, China). The rats (n = 35) were individually housed in cages for 3 days at 23 ± 2°C with a 12-hour light/dark cycle and free access to standard rat chow and water. All rats were anesthetized by intraperitoneal administration of 10% chloral hydrate (4 mL/kg) and fixed on a stereotaxic apparatus (Kopf Co., Tujunga, CA, USA). The target areas were bilateral brain ventricles (1.1 mm posterior to bregma; 1.5 mm lateral to the midline bilaterally; 4 mm below the dura) (Paxinos et al., 2005).

The study was approved by the Animal Care and Use Committee of Central South University of China (approval No. 2016-015). The experimental procedures followed the Guide for the Care and Use of Laboratory Animals (United States National Institutes of Health Publication No. 85-23, revised 1986).

Rat model and intervention

A rat model of AD was established by microinjecting with a microsyringe (5 μL per side, 1 μL/min) aggregated Aβ1-42 (Sigma-Aldrich, St. Louis, MO, USA) into the bilateral brain ventricles of rats. After injections, the needle was left in place for 5 minutes before it was slowly extracted. The skin incision was disinfected with complex iodine and sutured. All operations were performed under sterile conditions.

Rats in the sham-operated group (sham group) were treated with the same method and injected with the same amount of Aβ1-42 (Sigma-Aldrich). Both Aβ1-42 and Aβ1-25 were dissolved in sterile distilled water at a concentration of 2 mg/mL and incubated at 37°C for 1 week to form...
aggregations (Teng et al., 2014).

The rats were randomly divided into the five following groups (n = 7 per group): sham group (Aβ35-35 intracerebroventricular injection + distilled water), AD model group (Aβ35-35 intracerebroventricular injection + distilled water), low-dose BTD group (Aβ35-35 intracerebroventricular injection + low-dose BTD), moderate-dose BTD group (Aβ35-35 intracerebroventricular injection + moderate-dose BTD) and high-dose BTD group (Aβ35-35 intracerebroventricular injection + high-dose BTD).

**BTD preparation**
The BTD extracts (Herba Epimedi, Radix Polygoni multiflori, Plastrum Testudinis, Fossilia Ossis Mastodi, Radix Pogalae and Rhiyma Acorus tatarinowii at a ratio of 3:3:4:4:2:2) prepared for use as a crude plant medicine were purchased from the Pharmacy of Xiangya Hospital in Central South University of China and authenticated by the herbal medicinal botanist Professor Peng Lei at the Department of Chinese Herbal Medicine of Central South University (Changsha, Hunan Province, China). The extracts were immersed in distilled water for 1 hour, decocted for 1.5 hours, heated to the boiling point, and then filtered. The amount of distilled water was 10 times greater the amount of the medicine. The crude plant medicine was decocted again for 1 hour, and filtered through a strainer (pore size, 0.18 mm). The amount of distilled water was 8 times greater than the amount of the medicine. The filtrate was dissolved, condensed to a viscous liquid in a 45–65°C water bath and dried using a vacuum-asisted freeze-drying device (Min Jie Machinery Co., Shanghai, China). The samples were pulverized under aseptic conditions and the obtained lyophilized powder was stored at 4°C for later use.

**BTD treatment**
Three days after the intraventricular injections of aggregated Aβ, rats in the low-, moderate- and high-dose BTD groups received 0.563, 1.688, and 3.375 g/mL, respectively, (4 mL/day) of a BTD water suspension (10 mL/kg; dose ratio, 0.018) (Xia et al., 2017). Rats in the sham and model groups were daily administered 4 mL of distilled water via oral gavage. All rats were treated for 28 consecutive days.

**Morris water maze test**
The Morris water maze apparatus was a round stainless-steel tank (diameter 150 cm, height 100 cm). The pool was considered to have four equally spaced quadrants, with one of four figures (triangle, five-pointed star, diamond, and circle) marked on the wall above each quadrant. A platform (diameter 4 cm, height 58 cm) was placed in the center of one quadrant. The water (depth, 60 cm) was dyed black with nontoxic food color additives and was maintained at a temperature between 22 and 25°C. The swimming track of the rats was recorded using a video-tracking camera (Canon, Tokyo, Japan) positioned above the tank. During training, the objects around the pool were kept the same, and the indoor environment was quiet.

For the navigation test, each rat was placed in the water perpendicular to the pool wall from a specific point in each quadrant and trained four times each day for 60 seconds per trial for 5 days. The computer connected with the video-camera stopped recording as soon as the rat landed on the platform or when 60 seconds had lapsed. The rat was allowed to rest on the platform for 10 seconds. The amount of time spent finding the platform (escape latency) was calculated using Morris water maze software (Panlab Company, Holliston, MA, USA).

On day 6, a spatial probe test was conducted with the platform removed. The rat was placed in the water perpendicular to the wall from a selected position. The number of crossings over the location of the previously hidden platform and the time spent in that (target) quadrant were measured and recorded for 90 seconds by the Morris water maze software (Panlab Company). The investigator conducting the Morris water maze test was blinded to the treatment groups.

**Golgi staining**
All rats (n = 7 per group) were killed after 28 days of intragastric BTD (or water) administration. The Golgi–Cox impregnation of brain tissue was performed by FD Neurotechnologies, Inc. (Columbia, SC, USA), using the FD Rapid GolgiStain Kit (#PK401; FD Neurotechnologies, Inc.). Brains were immersed in impregnation solution, which was prepared by mixing equal volumes of Solutions A and B, and stored at room temperature in the dark. The solution was replaced with fresh impregnation solution the next day. After 2 weeks, the brains were transferred to Solution C and stored at room temperature for 72 hours. The solution was replaced after 24 hours. The brain tissue was sectioned into 100-μm-thick sagittal slices using a cryostat and were mounted on gelatin-coated microscope slides with Solution C. Slides were rinsed twice in distilled water (4 minutes each), and then placed in a mixture of one part Solution D, one part Solution E, and two parts double-distilled water for 10 minutes. The slides were rinsed twice in distilled water (4 minutes each), dehydrated in 50%, 75%, and 95% ethanol (4 minutes each), and further dehydrated in absolute ethanol four times (4 minutes each). Sections were permeabilized in xylene three times (4 minutes each) and coverslipped with Permount mounting medium (Thermo Fisher Scientific, Waltham, MA, USA). The dendritic spines on secondary and tertiary apical dendrites of pyramidal neurons in the CA1 region of the hippocampus were selected for quantitative analysis and quantified by two different investigators. The dendritic spine density values are equaled to spine numbers/dendritic length and expressed as spines/10 μm (Li et al., 2013).

**Western blot assay**
All rats (n = 7 per group) were killed after 28 days of BTD (or water) administration. Hippocampal tissues were collected and homogenized on ice. Cold phosphate-buffered saline (0.01 M, pH 7.2–7.3) was dropped onto the tissues. After gently shaking and washing, the solution was poured off. Western blot assays were conducted as previously de-
scribed (Zhang et al., 2015). Briefly, hippocampal tissues were lysed in radioimmunoprecipitation assay buffer (Applygen, Beijing, China). The protein content, measured using a bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA, USA), ranged from 2 to 4 μg/μL. All protein lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat milk for 1 hour at room temperature and incubated with the following primary antibodies (all obtained from Proteintech, Chicago, IL, USA) overnight at 4°C: mouse β-actin antibody (1:4,000), rabbit Shank1 polyclonal antibody (1:200), rabbit NR2B polyclonal antibody (1:200), and rabbit PSD-95 polyclonal antibody (1:200). Subsequently, the membranes were incubated with secondary goat anti-mouse antibody or rabbit IgG conjugated to horseradish peroxidase (1:3,000; Proteintech) for 1 hour at room temperature. Bound antibodies were visualized using the enhanced chemiluminescence western blotting detection kit (Thermo Pierce, Waltham, MA, USA). Proteins were visualized on X-ray film, and the grayscale values of the bands were analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA). Target protein expression was normalized to β-actin.

Statistical analysis
The data are expressed as the mean ± SD and were analyzed with SPSS 21.0 software (IBM, Armonk, NY, USA). Statistical analysis of the escape latency was performed using a repeated-measures analysis of variance (ANOVA). Other data were analyzed with one-way ANOVA followed by least significant difference tests. Values of P < 0.05 were considered statistically significant.

Results
BTD ameliorated Aβ25-35-induced learning deficits
The navigation test results indicated that the escape latency for all rats significantly decreased with time (P < 0.01; repeated measures ANOVA), suggesting that training was effective. However, the escape latency among the groups differed (P < 0.01). Compared with the sham group, the group administered Aβ25-35 had a significantly longer escape latency (P < 0.01), indicating that administration of Aβ25-35 induced learning deficits. This deficit was ameliorated by the administration of both moderate and high doses of BTD (P < 0.05 or P < 0.01; Figure 1A, D).

BTD alleviated Aβ25-35-induced memory deficits
The results of the spatial probe test demonstrated that compared with the sham group, rats in the model group had significantly fewer platform crossings (P < 0.05) and spent less time in the target quadrant (P < 0.01). These indexes reflecting retention of memory in rats revealed that Aβ25-35 induced memory deficits. However, BTD ameliorated the Aβ25-35 induced memory deficits in a dose-dependent manner, significantly increasing the number of platform crossings (P < 0.05 or P < 0.01) and the time in the target quadrant (P < 0.05 or P < 0.01) in AD rats compared with those in the model group (Figure 1B, C). These results indicated that BTD had positive effects on spatial memory.

Long-term oral administration of BTD inhibited hippocampal synaptic loss in a rat model of AD
We assessed the density of dendritic spines on pyramidal neurons using Golgi-stained tissue. The density of the dendritic spines on pyramidal neurons was markedly decreased in the model group compared with that in the sham group, and this decrease was significantly blocked by moderate-dose BTD treatment (Figure 2).

BTD increased PSD-95, Shank1, and NR2B hippocampal protein levels in a rat model of AD
To determine the effect of BTD on synaptic plasticity-associated proteins, we performed western blot assays. As shown in Figure 3, PSD-95 (P < 0.05), Shank1 (P < 0.01) and NR2B (P < 0.05) protein expression levels were significantly decreased in the model group compared with those in the sham group. However, BTD administration for 28 days significantly increased protein expression levels in the treated rats compared with that for rats in the model group for PSD-95 (P < 0.05 or P < 0.01) and Shank1 (P < 0.05 or P < 0.01) at all three doses and for NR2B at both the moderate and high doses (P < 0.05 or P < 0.01; Figure 3).

Discussion
Our findings demonstrated that the abnormal deposition of Aβ in the brain is neurotoxic and is associated with synaptic damage in the hippocampus. Soluble Aβ oligomers, rather than Aβ plaque or neurofibrillary tangle volume, reportedly induces synaptic loss (Zhang et al., 2015). Previous studies have shown that both Aβ1-42 and Aβ1-40 peptides can induce synaptic injury and are implicated as playing a role in AD (Bate et al., 2008; Lazcano et al., 2014). However, many researchers have used the smaller 11-amino acid fragment, Aβ1-35, as a convenient alternative in AD investigations, rather than the parent Aβ1-42, as the smaller peptide mimics several of the toxicological and oxidative stress properties of the native full-length peptide (Frozza et al., 2009; Zheng et al., 2016). The methionine at the C-terminus in Aβ1-35 appears to be the cause for the exaggerated effects of this peptide (Varadarajan et al., 2001). Aβ has been shown to decrease synaptic N-methyl-D-aspartic acid receptor-induced long-term potentiation and long-term depression (Minano-Molina et al., 2011). These results are consistent with data in Wistar rats showing that spatial memory performance induced by flavonoid intervention significantly correlates with the hippocampal levels of the NR2B glutamate receptor subunit (Rendeiro et al., 2014). It was previously demonstrated using immunohistochemistry that Aβ interacts with PSD-95 at synaptic sites (Pham et al., 2010). Roselli et al. (2009) found that shank synaptic clusters, which are associated with the ribosomal s6 kinase signal pathway, markedly decrease in frontocortical neurons treated with soluble Aβ. Therefore, soluble Aβ oligomers play a dominant role in the beginning
of AD and can cause synaptic dysfunction and cognitive decline (Shankar et al., 2008).

Alzheimer’s disease, a complex and heterogeneous disorder, still lacks effective prevention and treatment methods. Traditional Chinese medicine may offer certain benefits in the prevention and treatment of AD, given that pathogenesis occurs with myriad symptoms and features damage to multiple systems that affect the entire person. Although BTD is composed of six traditional Chinese medicines, Herba Epimedii and Radix Polygonum multiflori are the main effectors. They nourish the kidney, replenish the blood and essence, and antagonize the neurotoxicity of Aβ to improve learning and memory abilities (Zhang et al., 2014; Park et al., 2015), while the other ingredients in BTD provide additional favorable effects.

The present study investigated the effects of BTD on postsynaptic proteins to determine the mechanism underlying the amelioration of the memory deficits. We hypothesized that the improvements in the spatial learning and memory abilities in this rat model of AD were associated with an inhibition in the deficit-associated decrease in the expression levels of the synaptic plasticity-associated proteins Shank1, NR2B, and PSD-95. We found that not only measures of memory impairment were significantly improved after BTD treatment but also protein expression levels of Shank1, NR2B, and PSD-95 were significantly increased. Icariin (the
active chemical constituent in Epimedium) reportedly increases neuronal cell activity, preserves the expression levels of synaptic protein PSD-95, reduces Aβ plaque deposition, and protects against mitochondrial fragmentation. The present data support the results of our recent study in C57BL/6J mice showing that Polygonum multiflorum has inhibitory effects on inflammatory mediators and microglial activation, both of which are associated with the attenuation of memory impairment (Park et al., 2015). These previous reports together with our current results suggest that Epimedium and Polygonum multiflorum have multiple targets and functions that may offer desirable advantages against multifactorial neurodegenerative process and diseases, including AD.

In conclusion, BTD may block Aβ-induced neurotoxicity by regulating the protein expression levels of PSD-95 and Shank1 and the NR2B signaling pathway to maintain synaptic structure and transmission efficiency. Although the exact molecular targets of BTD that affect synaptic proteins and protect against cognitive decline are unknown, our study provided preliminary data to shed light on the underlying molecular mechanisms.

**Author contributions:** ZW, CXS, and SH designed and supervised the whole experimental process. WG and SC performed experiments. SH and WJP provided technical assistance and statistical analysis and reviewed and edited the paper. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Research ethics:** The study was approved by the Animal Care and Use Committee of Central South University (approval No. 2016-015). The experimental procedure followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1986).

**Data sharing statement:** Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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