Effect of Bushenyisui Formula on brain tissue apoptosis and Bcl-2 in beta-amyloid protein-induced Alzheimer's disease rat models

Shuke Cui, Yan Sun, Chengzang Liu

Abstract

OBJECTIVE: To investigate the effect of Bushenyisui Formula on cell apoptosis and positive B cell lymphoma (Bcl-2) in the Brain of rat models of Alzheimer's disease (AD) induced by beta-amyloid protein (Aβ) and the mechanism underlying the effect.

METHODS: Total of 40 SD rats, 20 females and 20 males, were randomly assigned to 4 groups, controlled group (A), model group (B), conventional treatment group (C) and Bushenyisui Formula treatment (BYFT) group (D), 10 rats in each group. Aβ1-42 was injected into the bilateral hippocampus of the rats in group B, C and D to create the models of AD. Sham operation was performed on the rats of group A in the same way by injecting equal volume of 0.9% sodium chloride solution into their bilateral hippocampus. 5 days after operation, Bushenyisui Formula was intraperitoneally administered at a dose of 450 mg/kg to the rats of group D (QD) for 20 days. Equal volume of 0.9% sodium chloride solution was intraperitoneally injected into the rats of group B with the same procedure. C suspension (20 mg/mL) was intraperitoneally injected into the rats of group B with the same procedure. The number of apoptotic cells in Brain and the positive Bcl-2 were counted. The changes of learning and memory abilities were evaluated using Y-maze.

RESULTS: Right after the establishment of the models, group B, C and D compared to group A respectively, the outcomes of Y-maze were significantly different from that of group A, which suggested obvious learning and memory disorder in those groups (P<0.01). After treatment, the times of electronic shocks of group C and D were significantly less than that of group B (P<0.05), and the numbers of apoptotic cells and positive Bcl-2 were significantly different from those of group B, apoptotic sells' number of group C and D smaller than that of group B and the number of positive Bcl-2 greater than that of group B.

CONCLUSION: Bushenyisui Formula could increase the number of Bcl-2 in brain, which improved the function of nervous system pertaining to learning and memory abilities.

© 2012 JTCM. All rights reserved.

Key words: Alzheimer disease; Apoptosis; Genes, bcl-2; Bushenyisui Formula

INTRODUCTION

Alzheimer's disease (AD) is a slowly progressive illness characterized by cognitive, personality and behavioral changes. The first symptom is recent memory impairment, and then, followed by a series of cognitive dys-
function including decline in language ability, the loss of reasoning ability, movement impairment, personality change, and so forth. The main pathology features in brain atrophy, deposits of senile plaques and neurofibrillary tangles in the brain.

Due to the complexity of AD’s pathogenesis it brings a great difficulty for its treatment. So seeking a way to combat it has been a task for medical science that is not only able to improve the cognitive function of AD suffers comprehensively, but also fit for long-term use with little side effects. Bushenyishou Formula, a Traditional Chinese Medicine (TCM) treatment, has bidirectional regulation effect on AD without obvious toxic and side effects and caught the attention of medical professionals.

Recently, quite a few studies have reported on the mechanism of treating AD with TCM. They mainly focus on the regulation of central neurotransmitters, antioxidant and anti-neurotoxin properties. However, the research into TCM treatment of this disease is not yet systematic and far from comprehensive. Therefore it is very imperative to exploit advanced molecular biological methods, from the perspectives of genomics and proteomics, to further study TCM’s effect on AD.

Focusing on apoptosis, the study compared the animals’ learning and memory abilities before and after treatment, explored the effect of BuShen YiSui Formula on cell apoptosis and Bcl-2 expression in the rat models in the hope for providing theory basis for the treatment of AD.

MATERIALS AND METHODS

Animals
Forty purebred SD rats, 300-350 g, half females and half males, were supplied by the Experimental Animal Center of Medical School of Xi’an Jiaotong University. Animal certificate No. is 08-005.

Reagents
Bcl-2 detection kit was purchased from Beijing Zhongshan Biological Technology Limited Company. Apoptosis detection kit was purchased from Beijing Zhongshan Biological Technology Limited Company.

Main instrument
Stans equidistant-radial-arm Y-maze was supplied by the Department of Pharmacology of Shaanxi University of Chinese Medicine; C-type stereotaxic instrument was supplied by Huaibei Zhenghua biological equipment limited company. The TL-2000MM-III micro-oscillator was supplied by Jiangsu Jiangyan Tianli Medical Instrument Limited Company. E200 Nikon microscope was supplied by Nikon Nanjing Jiangnan Optical Instrument Company Limited. Small tank was supplied by Beijing Medical Equipment Factory. GTK-2002 automatic thermostat was supplied by Xi’an Ruifeng Equipment Company.

Animal grouping and model creating
Forty purebred SD rats were fed for three days for adaptability purpose, males and females separated in different cages with natural light, drinking and eating freely. Then they were randomly divided into four groups, 10 for each. They were control group A, model group B, conventional treatment group C and BYFT group D. Each rat of group B, C and D group, after anesthetized with 10% chloral hydrate (0.5 mg/kg), was placed on stereotaxic instrument, then the anterior fontanelle located and then skin covering it was cut open. Further, by referring to rat anatomy atlas, the hippocampus’ projection on body surface was located at 3.0 mm away behind anterior fontanelle and at 2.0 mm away from the rat body’s midline. A dental drill was used to drill a hole into the skull, then a micro-syringe was pinned on the stereotaxic apparatus and needed to the depth of about 2.8 mm, then 10 μg/5 μL of Aβ1-42 solution (fiber type) was slowly injected into the skull and let the needle stay for 5 min after finishing the injection, then slowly removed the syringe. The rats of group A underwent the same operation except for injecting 5 μL of saline.

Treatments of the rats
Treatments started on the fifth day after the operations mentioned above. The rates of group A and B were kept in separate cages with natural light and freely accessed to water without any other special treatments. In group C, piracetam (lot number: 031203; Shanghai Huashi Pharmaceutical limited company) was used to prepare the suspension of 20 mg/mL with warm boiled water. Each rat was intragastrically administered the suspension with the dose of 450 mg/kg per day (about 2 mL for each rat) group D (QD) for 20 days.

In group D, each rat was intragastrically administered the decoction of Bushenyisui Formula with the dose of 450 mg/kg per day QD for 20 days. The prescription of the formulas was: rhizoma Ligustici wallichii 10 g, notoginseng 10 g, saffron 10 g, turmeric 10 g, rhizoma gaostrichia 10 g, leeches 10 g, dried tangerine peel 10 g, rhizoma acori tatarinowii 10 g, salvia 20g, astragalus 30 g, atractyloides 15 g, rhubarb 6 g, amomum 6 g.

Data collection
Y-maze test was conducted for all rats in group A, B, C and D at one day before the model-creating operation, the fifth day after the operation, and at the day after treatments finished. After the tests, the rats were fasted for 12 hours, and then specimens and other data were collected.

Y-maze test: Equidistant-radial-arm Y-maze was used to measure space learning and memory abilities of the rats. They had two min to adapt to the environment of Y-maze. One arm was chosen as starting point where electric shock was given to the rats staying there (30-50V, 0.5-0.7mA, delayed for 10 s) to make them escape to a safe area. After one-min break, the process...
was repeated. Counts of which they were electrically shocked were recorded. 9 out of 10 times that they made correct responses meant that they had learned the lesson. The counts were used to evaluate their learning and memory abilities.

Specimen obtaining: 3% pentobarbital solution (30 mg/kg) was intraperitoneally administered for anesthesia purpose, then cardiac perfusion was performed, brain tissue striped, and slice was cut from the coronal section of hippocampus and fixed with 4% paraformaldehyde phosphate buffer and water was eluted with gradient ethanol, then it was clarified with xylene. Finally it was conventionally embedded with paraffin wax.

Bcl-2 and cell apoptosis detection: 1) Immunohistochemistry ABC method for Bcl-2. Making paraffin section of paraffin-embedded slice and washing it in 0.01 Midkine Phosphate Buffer Solution (MKPBS) three times, 5 min each time. Adding 0.3% solution of hydrogen peroxide in methanol (methanol 80 mL + 0.01 MKPBS 100 mL + 30% hydrogen peroxide) and soaking them for 30 min to eliminate the effect of the endogenous peroxidase, then washing them in 0.01 MKPBS again three times, 5 min each time. Adding 0.3% Triton X-100 (polyethylene glycol octylphenyl ether) and soaking it for 30 min, to increase cell permeability, and washing it in 0.01 MKPBS once more three times, 5 min each time. Adding serum dilution (1 g rat serum albumin + 0.01 MKPBS 100 mL + 0.08 g sodium azide) to primary antibody, storing the diluted primary antibody at temperature of 4℃ for 24-48 h, absorbing the antibody, and then washing it in 0.01 MKPBS again three times, 5 min each time. Adding 0.01 MKPBS to the diluted secondary antibody, incubating it at room temperature for 2 h, then washing it in 0.01 MKPBS three times, 5 min each time. Adding rat tumor necrosis factor complex antibody and incubating it at room temperature for 2 h, then washing it with 0.01 MKPBS three times, 5 min each time, and then washing it with distilled quickly three times; Adding color development solution for immunohistochemical staining, once the cells were stained and the color of cell background became lighter, eliminating the color development solution immediately, and washing it quickly with distilled water three times, then adding 0.01 MKPBS to terminate further reaction.

After dehydrating it with gradient alcohol, sealing it and viewing it with microscope and record the results.

2) Apoptosis TUNEL assay: Placing the tissue slice in staining jar, washing it twice with xylene, 5 min each time, then washing it twice with anhydrous ethanol, 3 min each time; washing it with 95% ethanol and 75% ethanol respectively, 3 min each time; washing it with phosphoric buffer solution (PBS) for 5 min, then adding proteinase K solution (20 ug/mL) and letting it be hydrolyzed at room temperature for 15 min, then removing histone; washing it with distilled water 4 times, 2 min each time.

Adding 2% PBS containing hydrogen peroxide into the staining jar, letting them to react at room temperature for 5 min; washing the slice twice with PBS, 5 min each time.

Absorbing with filter paper the excess liquid surrounding the slice; adding immediately 2 drops of terminal deoxynucleotidyl transferase (TDT) buffer, and letting it to stand at room temperature for 5 min.

Again absorbing with filter paper the extra liquid surrounding the slice; adding immediately 54 ul TDT reaction mixture and putting it in a humidified chamber at 37℃ for 1 h for reaction.

Placing it in staining jar and adding reaction termination buffer preheated to 37℃ for washing purpose; keeping the temperature of 37℃ for 30 min, moving the slice up and down every 10 min so as to gently stir the liquid.

Washing it 3 times with PBS, 5 min each time, then adding two drops of peroxidase-labeled anti-digoxin antibody and placing it into humidified chamber for 30 min for reaction.

Washing it with PBS 4 times, 5 min each time, then adding the freshly prepared 0.05% diaminobenzidine (DAB) solution and letting it to be stained at room temperature for 3 min-6 min.

Washing it with distilled water 4 times, 1 min each time for the first three times, 5 min for the fourth time. Restaining it with methyl chloride at room temperature for 10 min.

Washing it with distilled water 3 times, 1 min each time. For the first two times after washing it, moving the slide up and down for 10 times; for the last time after washing it, letting it stand still for 30 s; then washing it three more times using 100% N-Butanol with the same procedure. Dehydrating it three times with xylene, 2 min each time, then sealing the slice. After drying it, viewing it with microscope and recording experimental outcome.

Statistical analysis
SPSS 13.0 was used for data analysis. T-tests were performed to compare group difference, P<0.05 was the significant level.

RESULTS

Y-maze test results
The Y-maze test results are showed in Table 1. The means ± SDs were summarized from the times they required for practice until they attain the goal that 9 out of 10 times they would escape to a safe area without being electrically shocked.

The detection of cell apoptosis and positive Bcl-2
The counts of apoptotic cells and positive Bcl-2 are shown in Table 2.
Notes: a: Group B, C and D were compared with group A respectively. P values were all smaller than 0.01 (P<0.0001, P<0.0001, P<0.0001); b: after treatment, group C and D were compared with group B respectively. P<0.05 (P<0.0365, P<0.0182).

**DISCUSSION**

The application of transgenic technology recent years in the research work of animal models of AD strongly supports the theory that AD is caused by Aβ. There were more reports on AD model induced by injecting Aβ into hippocampus at home and abroad. It was a practical choice for the research into AD. Recent studies showed that apoptosis was one of the important causes that decreased the number of neurons in brain tissue of AD. Apoptosis is controlled by genes and the process is the result of direct signal transduction. The pharmacological effect of piracetam improves the disturbance of consciousness caused by traumatic brain injury, stroke, encephalitis, poisoning, and improves learning and memory ability, and chronic brain dysfunction syndrome, the symptoms of symptomatic psychosis, psychogenic symptoms and extrapyramidal symptoms induced by medicines etc. It was widely used as a conventional treatment in cerebrovascular diseases. The use of piracetam can largely catch up with recent clinical practice.

There is no name of AD in ancient medicine, but dementia was discussed as early as 2000 years ago in some literature such as Tao Chuan and Huang Pu Mi’s Zhenjiu Jiayi Jing. I believe that, from the perspective of TCM, AD’s etiology and pathogenesis are complicatedly associated with the imbalance of Yin and Yang, Qi and blood, and Zangfu’s function. Mostly it is due to ageing, which causes the deficiency of Zangfu’s Qi and blood. Although the disease locates in the brain, it is related to heart, liver, spleen and kidney. Of them the weakness of kidney function which causes the dysfunction of brain is AD’s fundamental pathogenesis. To sum up, the AD pathogenesis, in terms of TCM theory, can be describe as deficiency is the root cause while excess pattern is manifested. The deficiency results from aging that leads to the deficiency of Qi, blood, Yin and Yang and the deficiency of several Zangfu organs. Of the deficient organs, kidney is the most deficient one. The combined conditions cause the incapability to produce enough Qi, blood and essence etc to nurture the brain, then its marrow becomes deficient, thus the original spirit of life lacks nourishment, the bright spirit becomes blunt. Phlegm and blood stasis are the pathological products of Zangfu organs’ deficiency. Due to the deficiency of heart liver, spleen and kidney, the distribution of Qi, blood and body fluids is disordered, thus phlegm and blood stasis generated from inside block the meridians and collateral vessels, and blunt the brain, thus mental confusing ensues. In the formula, rhizoma Ligustici can reduce concentration of platelet thromboxane A2 and raise the level of prostaglandins while inhibiting vascular smooth muscle contraction, increase blood flow of cerebral arteries. Salvia has the effect of inhibiting the aggregation of thrombus and blood clots, and significantly dilating microvascular vessels, and scavenging oxygen free radicals, blocking calcium channels, regulating the hemato-crit, and so forth. Leech contains hirudin, heparin, anti-thromboxane. It can dilate blood vessels, promote blood circulation, and inhibit the effect of thrombin on fibrinogen increase high-density lipoprotein as well. Rhubarb can shorten the time of blood coagulation, reduce capillary permeability for fibrinogen, and improve lipid metabolism disorder. Modern pharmacological study also showed that astragalus, notoginseng, rhizome gastodaria, saffron, turmeric, radix curcumae and rhizoma acori Tatarinowii had the effects of anti-free radical damage, anticoagulant, anti-platelet aggregation, promoting fibrinolysis, dilating blood vessels etc and that angerine peel, amomum and atractylodes seng, rhizome gastrodia, saffron, turmeric, radix curcumae and rhizoma acori Tatarinowii had the effects of anti-free radical damage, anticoagulant, anti-platelet aggregation, promoting fibrinolysis, dilating blood vessels etc and that angerine peel, amomum and atractylodes

### Table 1 The outcomes of Y-maze test (frequency, $\bar{x}$ ±s)

<table>
<thead>
<tr>
<th>Time</th>
<th>Group A $\bar{x}$ ±s</th>
<th>Group B $\bar{x}$ ±s</th>
<th>Group C $\bar{x}$ ±s</th>
<th>Group D $\bar{x}$ ±s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before operation</td>
<td>15.5±2.4</td>
<td>15.0±2.5</td>
<td>16.0±3.1</td>
<td>17.3±21.0</td>
</tr>
<tr>
<td>After operation</td>
<td>15.0±2.5</td>
<td>26.1±6.7$^c$</td>
<td>25.2±4.0$^c$</td>
<td>23.3±5.8$^c$</td>
</tr>
<tr>
<td>After treatment</td>
<td>14.8±32.0</td>
<td>23.0±4.9</td>
<td>19.0±2.8$^c$</td>
<td>19.3±3.8$^c$</td>
</tr>
</tbody>
</table>

Notes: a: Group B, C and D were compared with group A respectively. P values were all smaller than 0.01 (P<0.0001, P<0.0001, P<0.0001); b: after treatment, group C and D were compared with group B respectively. P<0.05 (P<0.0365, P<0.0182).

### Table 2 The outcomes of apoptotic cells and positive Bcl-2 detection ($\bar{x}$ ±s)

<table>
<thead>
<tr>
<th></th>
<th>Group B $\bar{x}$ ±s</th>
<th>Group C $\bar{x}$ ±s</th>
<th>Group D $\bar{x}$ ±s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>9.8±2.7</td>
<td>7.2±2.3$^c$</td>
<td>7.2±14.0$^c$</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>10.2±1.5</td>
<td>11.8±17.0$^c$</td>
<td>12.64±1.2$^c$</td>
</tr>
</tbody>
</table>

Notes: a: group C and D were compared with group B respectively. P values were all smaller than 0.05 (P<0.05, P<0.00157); b: group C was compared with group B. P<0.05 (P<0.0197); c: group B was compared with group D. P<0.05 (P<0.0009); d: group C was compared with group D. P>0.05 (P>0.1838).
by improving AD’s declined learn and memory abilities to some extent. However, in this study, the effect of Bushenyisui Formula on increasing positive Bcl-2 and reducing number of apoptotic neuronal cells were not significant different from those of piracetam. The study revealed that Bushenyisui Formula worked on both cell apoptosis and Bcl-2. The mechanism might be that the formula has effect on these two pathways or on an interactive pathway between them. Further study is required to further explore the mechanism underlying the formula treating AD.

REFERENCES

14 Guan XM, Han JS. Medical Neurobiology Beijing People’s Health Publishing House 2002; (7): 458.