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Basic Investigation

Effects of Compound Danshen tablets (复方丹参片) on spatial cognition and expression of brain β-amyloid precursor protein in a rat model of alzheimer's disease

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Abstract

OBJECTIVE: To observe the effects of Compound Danshen Tablets (CDST) on spatial cognition and expression of brain b-amyloid precursor protein (β-APP) in a rat model of Alzheimer's disease.

METHODS: The rat model of Alzheimer's disease (AD) was established using D-galactose to cause subacute aging combined with Meynert nucleus damage. Rat behavior was monitored using the Morris water maze, and the expression of β-APP in rat brain tissue was detected via immunohistochemistry.

RESULTS: CDST significantly improved spatial cognition and decreased β-APP expression in the cortex and hippocampus (P<0.05, P<0.01).

CONCLUSIONS: CDST can significantly improve spatial cognition in a rat model of AD. This observation is possibly related to a reduction in β-APP expression in the rat brain.

Key words: Compound Danshen Tablets (CDST); Alzheimer's disease; B-amyloid precursor protein

INTRODUCTION

Senile dementia mainly includes Alzheimer's disease (AD) and vascular dementia (VD). Alzheimer's disease is a primary, retrograde, brain nerve affliction induced by multiple factors. AD has become one of the main diseases severely harming senile health and influencing quality of life. At present, no effective preventive methods or cures are available for this disease. Compound Danshen Tablets (复方丹参片, CDST) are composed of Danshen (Radix Salviae Miltiorrhizae), Sanqi (Radix Notoginseng), and Bingpian (Borneolum). These tablets are indicated for heart disease and angina pectoris. Interestingly, our previous study demonstrated that the Baiyunshan brand Compound Danshen Tablets was able to greatly improve learning and memory in the experimental AD rat and decrease the amount of the excitatory amino acid, glutamine, in the brain, as well as lower the production of b-amyloid precursor protein (b-APP) [1,2]. In this study, effects of CDST on spatial cognition and expression of brain b-APP in experimental AD rats were further explored to investigate the ability of CDST to prevent and treat AD.

MATERIALS AND METHODS

Drugs and reagents

Shishanjian jia Tablets (Red Flag Pharmaceutical Factor of Shanghai Medical University, Shanghai, China, lot No.: 020603) and Compound Danshen Tablets (Guangzhou Baiyunshan Hutchison Whampoa Chinese Medicine Co. Ltd. Guangzhou, China, lot No.:

030435), were ground into powder, strained in a 100 mesh screen, dissolved in fresh distilled water and then prepared as a 6.3% water solution and kept at 4°C before use. D-galactose, packed by Amresco Inc. (Solon, OH, USA); Ibotenic acid (IBO), produced by Sigma-Aldrich Co. (St. Louis, MO, USA); IL-6, IL-2, and TNF-α radioimmunoassay kits were purchased from the Science and Technology Development Center of PLA General Hospital (Beijing, China). Rabbit -APP antibody, secondary antibody and Streptavidin/Peroxidase (SP) kits were purchased from Wuhan Boster Biotechnology Co. Ltd. (Wuhan, China).

Experimental animals

Sprague Dawley (SD) rats, SPF grade, weighing 300-380g, were supplied by the Experimental Animal Center, The First Military Medicine University. Number of certificate of quality: [Yue] 2002A040.

Instruments

The SN-2 stereotaxic instrument for rats were produced by Narishige Inc. (Tokyo, Japan); 307-6 Dental Engine, Shanghai Medical Analytical Instrument Factory (Shanghai, China). High speed refrigerated centrifuge was purchased from Beckman Inc. (Brea, CA, USA), the ultrasonic cell breaker from Ningbo Xinzhi Biological Science and Technology Co., Ltd (Ningbo, China), and the SN-695B γ–counter from Shanghai Rihuan Instrument Factory (Shanghai, China). The PYX water-separated electric heating thermostat incubator and the HZ-22s water bath thermostat agitator were obtained from Taicang City Science and Education Equipment Factory (Taicang, Jiangsu, China), the inverted microscope from Chongqing Optic Instrument Factory (Chongqing, China) and the CX41 Olympus imager analytical system was from Olympus Corporation (Tokyo, Japan).

AD Model

Ten SD rats were allocated to the operation control group, Fifty rats, 25 males and 25 females, were administered with D-galactose (intraperitoneal; 48 $mg \cdot kg^{-1} \cdot d^{-1}$) for 6 consecutive days. From the 7th day, after anesthesia, the rats were fixed on the stereotaxic instrument and a vertical cut in the middle of the vertex skin was made. The parietal bone was explored, and one small hole on each side was drilled posterior to the coronal suture. Location coordinates were: 0.9 mm behind the anterior fontanelle, 2.6 mm lateral to the mid line, 7.5 mm in depth. Each side was injected with 1 μL (containing IBO 5 μg/μL) at 0.2 μL/min by a microinjector, and the needle was retained for 10 min. Rats in the operation control group underwent the same operation, but were injected with an equal volume of saline. After the operation, the skin was sutured and penicillin (intramuscular) was administered within 3 days for infection prophylaxis[3,4]. Thirty-one surviving rats were randomly divided into 5 groups: operation control group, model group, Shishanjian jia group (2.3×10^{-5} g/kg), small dose CDST group (0.315 g/kg, corresponding to half an adult clinical dose), and large dose CDST group (0.630 g/kg, corresponding to an adult clinical dose). Animals were administered medications intragastrically for 2 months. Following this, a Morris water maze was used to ascertain behavior.

Morris water maze behavior experiment

Four water inlet points (R, P, L, O) were marked on the walls of the Morris water maze and the pool was equally divided into 4 quadrants. A platform was placed 1 cm below the water level in the middle of the pool. Morris water maze testing included place navigation for 5 days, 4 times each day. Rats were placed into the pool at the inlet points, facing the pool wall, and platform searching times within 2 min were recorded. If the rat was unable to find the platform within 2 min, the rat was drawn to the platform by the experimenter's hand, remained at the platform for 10 s, and was then placed in a cage. To determine spatial recognition, the platform was removed on the 6th day, and the rat was placed into the water pool from any one water inlet point. The times for searching out the platform over quadrants within 2 min were recorded.

Determination of IL-6, IL-2 and TNF-α content

Following behavioral studies, rats were killed and serum IL-6, IL-2, and TNF-α content were detected using radioimmunoassays.

Detection of b-APP positive cells in brain tissue

After paraffin imbedding, brains were cut into sections for H&E staining and immunohistochemical processing, with adjacent sections being used for these two kinds of staining. This allowed for comparison between similar sections following staining. 1) Sections were dewaxed; 2) and processed with 3% H_2O_2 water solution and protected from light for 30 min at room temperature. 3) Sections were then washed with DC distilled water, 1×2 min, 0.01M PBS, 2×5 min 4) and subjected to high pressure antigen repair. 5) Following this, sections were washed with 0.01M PBS, 3×5 min 6) and blocked with 1:10 normal sheep serum and incubated for 20 min at 37°C. 7) After blocking, rabbit anti-b-APP (1:100) was dripped onto the section, and after incubation for 30 min at 37° C, was allowed to stand at 4°C overnight. 8) The following day, sections were washed with PBS, 3×5 min 9) and diluted biotin-labeled secondary antibody (1:200) was dripped onto the sections and incubated for 30 min at 37° C, washed with PBS 3×5 min, 10) whereupon diluted horse radish peroxidase-labeled strepto-egg albumin (1:200; diluted with PBS) was added to the sections, incubated for 30 min at 37°C, and washed with PBS, 3×5 min. 11) Staining was developed with DAB for 5 min for each section. Sections were then fully washed with tap water, counter stained with hematoxylin, dehydrat-

ed, hyalinized, and mounted with neutral gum. Another negative control section was prepared with b-APP replaced by PBS. All other procedures were the same as the SP method. Five visual fields (200×) were observed for each section using a double blind method. Positive cells in the cortex and hippocampus were recorded and the mean was calculated, entered and stored in the OLYMPUS computer image analysis system, Olympus Co. (Tokyo, Japan).

Statistical Analysis: SPSS10.0 statistical software package was used for data processing. Analysis of variance was used for comparison of multiple groups. LSD or Tukey multi-comparison methods were used for homogeneity of variance and Tamhane's T2 multi- comparison method was used for in-homogeneity of variance.

RESULTS

Effects of CDST on spatial cognition in the rat AD model (see Table 1)

There were significant differences between the small dose CDST group and the large dose CDST group, compared with the model group (P<0.05), indicating that CDST significantly improves spatial cognition in the AD rat.

Effects of CDST on serum cytokines in the AD rat model (see Table 2)

As compared with the control group, serum IL-6 content in the model group significantly decreased (P< 0.05). By contrast when compared with the model group, TNF-α content significantly decreased and IL-6 content significantly increased in the large dose CDST group (both P<0.05).

Table 1 Effects of CDST on spatial cognition in the rat model of AD ($\bar{x}$ ±s)

Group	n	Dose (g/kg)	Time seeking the platform while crossing the quadrants			
			R	P	L	O
Control	10	—	9.40±2.67*	9.20±3.16**	6.80±2.90	5.40±2.12
Model	9	—	6.75±1.49	5.13±2.85	4.38±2.88	3.75±1.67
Shishanjian jia	7	2.3×10^{-5}	6.67±4.18	8.33±2.37*	6.00±4.05	4.83±3.37
Small dose CDST	8	0.315	9.50±4.07	8.25±3.24	6.50±2.27	6.00±2.98*
Large dose CDST	7	0.630	8.50±3.78	8.50±4.89	6.67±1.50*	5.50±1.87

Note: Compared with the model group,*P<0.05.

Table 2 Effects of CDST on serum cytokines in the rat model of AD ($\bar{x}$ ±s)

Group		Dose (g/kg)	TNF-α (ng/ml)	IL-2 (pg/ml)	IL-6 (pg/ml)
Control	10	—	1.641±0.513	3.685±0.574	210.0±50.2*
Model	8	—	1.785±0.214	4.169±0.902	138.2±54.8
Shishanjian jia	7	2.3×10^{-5}	1.539±0.474	3.154±1.075	130.3±41.3
Small dose CDST	8	0.315	1.646±0.302	3.885±0.465	171.6±55.6
Large dose CDST	8	0.630	1.557±0.211*	4.329±0.594	172.0±25.2*

Note: Compared with the model group,*P<0.05.

Table 3 Effects of CDST on brain b-APP expression in the rat model of AD ($\bar{x}$ ±s)

Group	n	Positive cell numbers in the cortex	Positive cell numbers in the hippocampus
Control	8	4.0±2.4*	14.4±4.3*
Model	9	9.5±4.3	19.9±5.9
Shishanjian jia	7	6.2±3.6	15.9±6.7
Small dose CDST	7	5.1±2.0*	14.3±4.6*
Large dose CDST	7	3.9±2.7**	12.1±4.9*

Note: Compared with the model group,*P<0.05.

Effects of CDST on brain b-APP expression in the rat AD model (see Table 3)

b-APP immunohistochemical positive neurons were mainly distributed in the brain cortex and hippocampus. Most of the neurons were different sizes and showed circular, ellipse and shuttle shapes. Positive product was mainly expressed in the cytoplasm. In the immunohistochemical negative control slide, the background was colorless and the nucleus was blue. In the operation control group, some cytoplasm in the rat brain showed light staining (brown). The cytoplasm showed darker staining in the model group with significantly more positive expression than the operation control group (P<0.05). In both the small dose CDST group and the large dose CDST group, the cytoplasm

showed light staining (brown), with significantly less positive cells than the model group (see Table 3).

DISCUSSION

AD is a progressive disease, characterized by senile plaques, amyloid sedimentation and nerve fiber twining in the brain. Presently, studies have also found that blood vessels in the brain of patients with AD also have changes, manifested as cellular atrophy of cerebral vascular smooth muscle cells and thinning of capillary endothelium[5]. Endothelial injury and sedimentation in brain tissues induced by β-amyloid (β-AP) are one of the important links inducing retrograde effects on neurons and the pathogenesis of AD[5].

At present, modern medicine still does not have specific effective therapy for senile dementia. Our study indicated that CDST could significantly improve spatial cognition in the experimental AD rat, and inhibit production of β-APP in the brain.

The pathogenesis of AD is very complicated. At present, there are many AD models with differing characteristics. In the past, IBO-injured central cholinergic neurons and their effects on animal behavior and biochemical indexes of the brain were thought to be similar to pathological changes in human AD. These models had shortcomings, such as the absence of the overexpression of APP and sedimentation of β-AP[3]. In recent years, Gao J, *et al*[4] found using immunohistochemistry, that β-APP is also over expressed in this model. Our study also confirmed that in the brain of the normal aging rat there was production of β-APP. In combination with induction by D-galactose, IBO further injured the nucleus basalis of Meynert. β-APP in rat brain significantly increased, with obvious spatial cognition disturbance, suggesting that this model has better reproducibility.

Compound Danshen Tablets (复方丹参片, CDST) are composed of Danshen (Radix Salviae Miltiorrhizae), Sanqi (Radix Notoginseng), and Bingpian (Borneolum). Danshen (Radix Salviae Miltiorrhizae) promotes blood circulation, removes blood stasis, enriches blood and tranquilizes the mind. Danshen (Radix Salviae Miltiorrhizae) mainly contains fat-soluble tanshinones and water-soluble danshensu and tanshinonic acids. Tanshinones can significantly improve learning and memory disturbances in the rat after injection of β-APP into the hippocampus[6], and also has anti-oxidative and anti-inflammatory activities. Tanshinonic acids also have significant protective activity on injured brain nerves[7]. Danshen (Radix Salviae miltiorrhizae) can also significantly improve spatial cognition disturbances in the rat with one-sided temporal lobe ischemic injury and the total tanshinonic acids can minimize cerebral ischemia by inhibiting release of glutamic acid[8]. Sanqi (Radix Notoginseng) can activate blood circulation, remove blood stasis, and replenish *Qi* and blood. Its main component is triterpenoid saponin, which is similar to ginsenoside. Both fresh and prepared Sanqi (Radix Notoginseng) have been shown to invigorate Qi and the blood. Total arasaponins have obvious protective action on experimental brain ischemia, and significantly improve memory disturbance induced by anisodine or $NaNO_2$ in the mouse. They have also been shown to have a significantly protective action on brain injury in another rat model of AD[9]. Bingpian (Borneolum) is an "assistant" drug in this formulation and has functions of causing resuscitation and restoring consciousness. Modern studies indicate that Bingpian (Borneolum) can improve the permeability of the blood-brain barrier[10], so as to increase the concentration of drug in the brain strengthening the pharmacological effect of the drug on nerves. In the past, CDST was mainly used for treatment of coronary artery disease and angina pectoris. Our results demonstrate that in the future CDST may play an active role in the prevention and treatment of cerebrovascular diseases and senile retrograde brain diseases.

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