Cognitive enhancement and neuroprotective effects of a traditional Chinese herbal compound medicine on $\text{A}\beta_{1-42}$ induced Alzheimer’s disease in rats

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Folia Neuropathol 2020; 58 (4): 365-376 DOI: https://doi.org/10.5114/fn.2020.102439

Abstract

Introduction: We aimed to explore the role of a novel traditional herbal compound medicine (HCM) in Alzheimer’s disease (AD).

Material and methods: 72 rats were randomized into control, AD, Donepezil and HCM groups. Injection of $\beta$-amyloid peptide ($\text{A}\beta_{1-42}$) into the lateral ventricle was used to induce AD in rats. Rats in treatment groups received HCM (1.5, 3.0 and 6.0 g/kg) and Donepezil (0.92 mg/kg) for 21 days, respectively. The spatial learning and memory ability were observed by Morris water maze (MWM) test. Haematoxylin-eosin (HE) staining was carried out for pathological morphology. The contents of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) in the hippocampus were determined using the spectrophotometric method. $\text{A}\beta$ expression was measured by immunohistochemistry and Western blotting.

Results: Rats in HCM groups spent less time to locate the platform and performed better in spatial learning and memory than the AD group ($p < 0.05$). Hippocampus in the HCM (6.0 g/kg) group had a complete pyramidal cell layer, in which the structure of morphology was normal and the number of neurons was larger than in the AD group ($p < 0.01$). The contents of SOD, CAT, GSH-Px were notably increased and MDA content was significantly decreased in the hippocampus in HCM groups than in the AD group ($p < 0.01$). The expression levels of $\text{A}\beta_{1-42}$ in HCM groups were markedly decreased than in the AD group ($p < 0.01$).

Conclusions: HCM has a protective effect on the learning and memory capacity in AD in rats, indicating that HCM had cognitive enhancing potentials on AD.

Key words: Alzheimer’s disease, $\beta$-amyloid, oxidative stress, hippocampus, traditional Chinese herbal compound medicine.

Introduction

Alzheimer’s disease (AD) is one of the most common age-related neurodegenerative diseases with a distinct neuropathology, which involves extracellular neurological $\beta$-amyloid peptide ($\text{A}\beta_{1-42}$) plaques and intraneuronal neurofibrillary tangles (NFT) and dementia attacking aged people [4,7,8].
AD patients are characterized by progressive and irreversible impairments in memory and cognition. With the increase in life expectancy, the number of AD patients is expected to increase from 35 million today to over 115 million by 2050 [40].

The self-aggregation and accumulation of extracellular Aβ and intracellular hyperphosphorylated tau (pTau) with cognitive impairment are defining features of AD [33]. The most typical pathological characteristic of AD was senile plaques (SP) due to the deposition of Aβ1–42 [23,26]. Although progress has been made in the diagnosis of possible AD [27], all clinical trials of interventions which aimed at slowing disease progression in patients with mild cognitive impairment (MCI) and AD have failed [16,30,32,38,43]. Therefore, an increasing focus is being put on prevention.

It is believed that pathological changes of the hippocampus may be pivotal for cognitive dysfunction, including learning and memory impairments [17,31]. It has been shown that superoxide anion, hydroxyl radical, hydrogen peroxide, and nitric oxide produce marked effects on neurodegeneration [2,14,25]. Oxidative damage induced by reactive oxygen species (ROS) has been implicated in the pathogenesis of neurodegenerative diseases [25]. Excessive production of ROS destroys protein, lipid and DNA [25,37]. Aβ1–42 could induce oxidative stress via ROS production, reactive nitrogen species, dysfunction of related antioxidant defence enzymes, cytokines and chemokines in neurotoxicity and neurodegeneration of AD [5,6,12,20]. Also, many studies demonstrated that AD related with the increase in oxidative stress in brain antioxidants has an important effect on animal models [21].

Traditional herbal compound medicine (HCM) consists of ten medicinal plants, including Adiantum capillus-veneris L., Alhagi pseudalhagi (Bieb.) Desv., Anchusa italica Retz., Cordia dichotoma G. Forst., Euphorbia maculata L., Foeniculum vulgare Mill., Glycyrrhiza glabra L., Lavandula angustifolia Mill., Melissa officinalis L., and Ziziphus jujuba Mill. It has already been applied to treat many age-related diseases, including memory dysfunction, behavioural and psychological symptoms of dementia. However, so far, there has been no academic report issued about experimental research on the therapeutic effect of HCM on AD. Consequently, we aimed to explore the effects of a novel traditional HCM on AD in rats.

Material and methods

Drugs and reagents

Aβ1–42 and bicinechonic acid (BCA) protein assay kits were bought from Sigma-Aldrich (St. Louis, MO, USA). All plant materials in HCM were obtained from Xinjiang Uighur Autonomous Region Traditional Uighur Medicine Hospital (Urumqi, China). Donepezil hydrochloride (110807A) was acquired from Weicai Pharmaceutical Co., Ltd. (Suzhou, China). Commercial kits for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), malondialdehyde (MDA), hematoxylin and eosin (HE), and 4% paraformaldehyde were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). SP Rabbit HRP Kit (DAB) was obtained from ZSGB-BIO Technology Co., Ltd. (Beijing, China). Chloral hydrate was got from Tianjin Chemical Reagent Factory (Tianjin, China). Anti-Aβ1–42 antibody (rabbit) (ab68896) was got from Abcam Technology (Cambridge, UK). β-Actin antibody (mouse) (CW0281), Goat Anti-Mouse IgG (H+L)-HRP (CW0102), Goat Anti-Rabbit IgG (H+L)-HRP (CW0103), ECL Western Blot Kit (CW0049) and Tissue Protein Extraction Kit (CW0891) were got from CWBIO Co. Ltd. (Beijing, China).

Preparation of the HCM extract

All plant materials in HCM were shown in Table I. A total of 10 kg powdered plant materials were immersed in 100 l dd-water at 80°C for 12 h and placed at 105 ±5°C for 2 h. The resulting extract was filtered through a 0.2-um filter after centrifugation (5000 × g for 15 min at 4°C). Then it was decompressed at 60 ±5°C and converted into a fine spray dry extract with 20.4% (w/w) yield in the vacuum drying device. The final dried extract was added to 0.5% sodium carboxymethyl cellulose (CMC-Na) solution.

Animals and protocols

Seventy-two SPF level male Wistar rats, aged 8-10 weeks old, weighing 250 ±20 g, were obtained from the Laboratory Animal Centre of Xinjiang Medical University. Rats were bred under standard laboratory conditions. All experimental protocols were approved by the Laboratory Animal Centre of Medicine Animal Care and Use Committee. Rats were randomized into control and AD groups. Anaesthesia
was induced by injecting 2% sodium pentobarbital (50 mg/kg) through the abdominal cavity. The heads of rats were set on the stereotaxic instrument with reference to the rat brain in Stereotaxic Coordinates [41]. 5 μl volume of aggregated Aβ1–42 (5 μg/μl) was injected by Hamilton micro-syringe within 10 min. Rats in the control group also had been injected equivalent sodium chloride in an identical manner. Postoperative penicillin was given (1 time/day) for the first 3 days in order to avoid infection. On the 4th day, rats in the AD group were randomly divided into the AD group, Donepezil group and HCM (1.5, 3.0 and 6.0 g/kg) groups, 10 rats per group. Rats in control and AD groups received distilled water (1 ml/100 g), rats in treatment groups received 1.5, 3.0 and 6.0 g/kg of HCM and Donepezil (0.92 mg/kg) for 21 days, respectively.

Behaviour test

Morris water maze (MWM) used for the behavioural task was performed to test spatial learning and memory ability of rats with minor modifications described in previous reports [22,28]. Morris water maze is a white circular pool (120 cm in diameter and 50 cm in height) with a featureless inner surface. During the test, the circular pool was filled with water in which 500 ml of milk had been mixed to a height of about 26 cm (23 ±2°C). The pool was divided into four quadrants of equal area (A, B, C, and D). A white platform (10 cm in diameter and 25 cm in height) was centred in quadrants C (target quadrant) of the pool and submerged 1 cm below the water surface so that it was invisible at the water level and there was nothing to directly show the location of the escape platform in and outside the pool so that rats must memorize the platform location in relation to various environmental cues. After receiving HCM for 21 days, rats were undergoing training for 5 days. On each day of training, every rat was given 60 s maximum to find the escape platform. When a rat failed to find the platform within 60 s, it was guided to the escape platform and placed on the platform for 10 s. Once reached the platform, the rat was allowed to stay there for 5 s. Swimming paths were recorded by image tracking software [36]. Latency time to find the escape platform was measured by using the analysis software.

The spatial exploration test was carried out after the orientation navigation test on day 6. Rats were put into the pool from the same point and the number of times that rats crossed the platform location, swimming distance in the target quadrant, entrance times to the target quadrant and swimming time in the target quadrant were recorded for each rat within a period of 60 s.

Collection of brain tissue sample

12 h after MWM test, rats were anaesthetized with intraperitoneal injection of 10% chloral hydrate (0.35 mg/kg) and transcardially perfused with 250 ml of phosphate-buffered saline (PBS) followed by 250 ml of 4% paraformaldehyde solution. Then, rats were sacrificed and whole brain tissues were quickly removed and rinsed with ice-cold isotonic saline.
The brains were fixed with 4% neutral paraformaldehyde at 4°C for 48 h, rinsed with running water for 24 h, and paraffin-embedded for HE and immunohistochemical staining after dehydration and hyaline. The other brains were stored at ~80°C for further analysis.

**HE staining of the hippocampal section**

The hippocampal section of the brain was stained by HE staining. Simply, slices were immersed in hemalum for 5 min, and rinsed with tap water, then eosin staining for 1 min. Sections were dehydrated in an ascending alcohol solution, made hyaline with xylene, and sealed. The pathological changes and numbers of neurons in hippocampus CA1 region were detected under Leica DM3000 Led optical microscopy (Germany) and analysed by MoticMed 6.0 Digital Medical Image Analysis System.

**Immunohistochemistry analysis**

The concentration of anti-Aβ_1–42 antibody was 1 : 1000. Immunohistochemistry staining was carried out as described in the instructions. PBS was chosen as a negative control. The results were analysed by an image acquisition and analysis system. The cytoplasm or the process in hippocampus (CA1) that was stained brown yellow was regarded as positive and was counted with MoticMed 6.0 Digital Medical Image Analysis System.

**Measurement of antioxidant activity**

Pieces of hippocampus tissues were homogenized with phosphate buffer (pH 7.4) containing 1 ml of diluted protease inhibitor at 4°C for 1 min. Then centrifugation was carried out at 10,000 × g for 15 min. Finally, the supernatant was used to detect the SOD, CAT and GSH-Px activity, and MDA level according to kit instructions. Bradford method was used for measurement of total protein concentration.

**Western blotting**

Total protein was extracted from small pieces of hippocampus tissue using Tissue Protein Extraction kit (CW0891, CWBiotech). Protein concentration was determined by BCA Protein Assay kit (CW0014, CWBiotech). All protein samples were separated by 12% separation gel electrophoresis (Hoefer, SE250, USA) at 120 V for 150 min. Then, the resolved protein was transferred onto PVDF membrane (Millipore) using wet-transfer method (Hoefer, TE250, USA) at 240 mA for 150 min. The membrane was washed with Tris buffered saline with 1% Tween-20 (TBST) and then blocked with 5% BSA (CW0049, CWBiotech) for 2 h at 37°C. It was then incubated with anti-Aβ_1–42 and anti-β-actin antibodies overnight at 4°C. Afterwards, the membranes were washed and incubated with goat anti-rabbit IgG conjugated to HRP secondary antibody for 40 min at 37°C. Immunoreactive bands were detected using an enhanced chemiluminescent kit (ECL, CW0049, CWBiotech). The blots were imaged using Fusion FX7 Chemiluminescence imaging system (Vilber, France) and quantifications were performed using the Image J v.1.44 software and normalized based on the intensity of the β-actin band.

**Statistical analysis**

Statistical analyses were performed using SPSS18.0. All data were expressed as means ± standard deviation (SD) unless otherwise indicated. Data of spatial learning ability in MWM task were analysed with two-way ANOVA repeated measure followed by a Dunnett’s post hoc-test for multiple comparisons. Data of the spatial exploration test were compared by one-way ANOVA, followed by Tukey’s post hoc-test or LSD. The value of \( p < 0.05 \) was considered as statistically significant.

**Results**

**Effect of HCM on spatial learning and memory ability**

A significantly shorter escape latency was observed in rats in HCM and Donepezil groups in comparison with those in AD group (\( p < 0.05 \) or \( p < 0.01 \)), as well as a significantly longer escape latency in AD rats when compared to those in the control group (\( p < 0.01 \), Fig. 1A). The representative tracks of all rats during the MWM maze task at day 5 were shown in Figure 1B.

A space exploration test was conducted to assess spatial memory capacity on the 6th day. The results showed a significant decrease in platform crossing time, swimming distance in the target quadrant, entrance times to the target quadrant and swimming time in the target quadrant in the AD group when compared to the control group (\( p < 0.01 \)). However, rats treated with HCM and Donepezil dis-
played a significant increase in all parameters when compared to the AD group ($p < 0.01$, Fig. 2).

**Effect of HCM on histopathological changes of the hippocampus**

Distinct cytopathological alterations of neurons were observed in hippocampus CA1 region from AD rats, including loss of neurons, degeneration of neurons and neuronal necrosis. The pyramidal cells were disordered, thinned, cell volume got shrunk, larger cell gap and irregular shape in the AD group in comparison with the control group. In contrast, there were only few of hippocampal CA1 neurons that displayed the cytopathological alterations in HCM (6.0 g/kg) and Donepezil groups in comparison with the AD group, which did not exist in control rats (Fig. 3A). The number of hippocampal neurons in CA1 region also analysed under an optical microscope and our data illustrated that $\text{A}_\beta_{1-42}$ injection caused a significant decrease in the number of neurons in hippocampal CA1 region in AD rats when compared to the control group ($p < 0.01$). However, HCM
(6.0 g/kg) and Donepezil treatment displayed a significant increase in the neurons number in hippocampus CA1 region when compared to the AD group ($p < 0.01$). The neurons number in hippocampal CA1 region in HCM (3.0 g/kg and 1.5 g/kg) groups was also increased, but the difference was not statistically significant ($p > 0.05$, Fig. 3B).

**Effect of HCM on the hippocampus oxidative stress level**

$A\beta_{1-42}$ injection significantly increased MDA content in hippocampus tissue in AD rats than in control group ($p < 0.01$). However, HCM treatment displayed a significant decrease in the hippocampal MDA level in a dose-dependent manner when compared to AD rats ($p < 0.01$, Fig. 4A). The MDA level in the Donepezil group was also significantly reduced than in AD group ($p < 0.01$). Both the activities of SOD and GSH-Px in hippocampus were significantly decreased in $A\beta_{1-42}$ induced AD rats than in controls ($p < 0.01$). HCM and Donepezil groups displayed a significant dose-dependent increase in the activities of SOD and GSH-Px when compared to the AD group ($p < 0.01$, Fig. 4B, C). The CAT activity in hippocampus was significantly decreased in $A\beta_{1-42}$ induced AD rats when compared to control rats ($p < 0.01$). However,
HCM (3.0 and 6.0 g/kg) and Donepezil groups showed an increased hippocampal GSH-Px level when compared to the AD group ($p < 0.01$, Fig. 4D). There was no significant difference in hippocampal CAT activity between the HCM (1.5 g/kg) group and the AD group ($p > 0.05$).

**Effect of HCM on Aβ$_{1-42}$ expressions in hippocampus tissue in Aβ$_{1-42}$ induced AD rats**

We detected Aβ$_{1-42}$ protein level in hippocampus tissue by the immunohistochemical method (Fig. 5A). Our results displayed that the mean count of Aβ$_{1-42}$ positive staining cells was significantly higher in hippocampus tissue in the AD group than the control group ($p < 0.01$). Compared with the AD group, the mean count of Aβ$_{1-42}$ positive staining cells was significantly decreased in hippocampus tissue in HCM groups and the Donepezil group ($p < 0.01$, Fig. 5B). Based on immunohistochemical examination results, the protein expression of Aβ$_{1-42}$ in hippocampus tissue was detected by Western blotting (Fig. 5C), and the results illustrated that the protein expression level of Aβ$_{1-42}$ in hippocam-
pus tissue of AD rats was significantly increased than control rats \((p < 0.05)\). Instead, compared with AD rats, there was a significant difference in the protein expression level of \(\text{A}\beta_{1-42}\) in hippocampus tissue in the Donepezil group and HCM groups \((p < 0.05)\).

**Discussion**

Alzheimer’s disease is a critical neurodegenerative disease characterized by degradation of memory and cognitive function with no effective treatment as yet. It is related with progressive and irreversible loss of cranial nerves, mainly in the cortex and hippocampus region \([1]\). The functional zones of hippocampus control memory and learning behaviour \([34]\). Progressive deposition of \(\text{A}\beta_{1-42}\) as a form of SP in hippocampus is the common pathological characteristics of AD \([39]\). \(\text{A}\beta_{1-42}\) promotes oxidative stress and lipid peroxidation in synaptosomes and neuronal cultures \([9]\). Accumulating evidence suggests that oxidative stress plays an important part role in the mechanism of \(\text{A}\beta_{1-42}\) induced neurotoxicity \([45]\). Oxidative stress is an imbalance state between the oxidant-antioxidant system caused by too much production of free radicals or a decline in antioxidant defences. It is caused by the higher metabolic demand and mitochondrial dysfunction, which is implicated in the development of AD \([11,19,44]\). \(\text{A}\beta_{1-42}\) could elevate oxidative damage to both func-
Fig. 5. Effect of HEM on the protein expression levels of Aβ_{1-42} in hippocampus of AD rats. A) The Aβ_{1-42} protein detected by the immunohistochemical method. B) The Aβ_{1-42} protein detected by Western blotting. ∆∆p < 0.01 vs. control group, and **p < 0.01 vs. AD group.

HCM, which is composed of ten medicinal herbs, has been widely used for treating patients with amnesia. Chemically, these herbal medicines are all rich in flavonoids, phenolics, glycosides, saponins, anthocyanin, tannins, triterpenoids, volatile oil as well as polysaccharides [3,15,18]. Our results provided first evidence for the possible neuroprotective mechanism of HMC on ameliorating Aβ_{1-42} induced learning and memory impairment in AD rats.

First, we investigated the effect of HCM on cognitive performance in Aβ_{1-42} induced AD rats by Morris water maze test. Compared with the control group, a significant longer escape latency was
demonstrated on AD rats. However, after treating with HCM, the escape latency in HCM treated rats was significantly shorter than in AD rats. Besides, a significant increase in the platform crossing time, swimming distance in the target quadrant, entrance times to the target quadrant and swimming time in the target quadrant were illustrated in HCM treated rats, while all those parameters were shown to be decreased in AD rats. All of behavioural test results suggested that HCM was effective in ameliorating cognitive deficits in Aβ1–42 induced AD rats.

Hippocampus is an important part of the brain limbic system and is closely related to learning and memory function of the brain, especially with spatial learning and memory function [13]. The intact structure and normal function of hippocampus are pivotal in maintaining the normal learning and memory of animals [34]. Our histopathological results showed that the neurons in hippocampal CA1 region in the AD group were severely damaged due to the intracerebroventricular injection of Aβ1–42, including destruction of cytoskeleton, loss of neurons, degeneration of neurons and neuronal necrosis when compared to the control group. However, HCM (6.0 g/kg) treatment can effectively prevent Aβ1–42 induced hippocampal CA1 neuronal damage, and only few of hippocampal CA1 neurons displayed the cytopathological alterations as compared to the AD group. The results indicated that HCM had a neuroprotective effect against Aβ1–42 induced neurotoxicity in AD rats. Therefore, we inferred that HCM can improve learning and memory ability in AD through protecting the neurons in the hippocampal CA1 region.

Increasing evidence supports that Aβ1–42 induced free radical and oxidative stress are pivotal in the neurotoxicity and pathogenesis of AD [10,42]. Some clinical research revealed that the MDA level of hippocampus tissue, cerebrospinal fluid and plasma have increased due to the peroxidation of lipid and protein caused by ROS in AD patients [24]. In addition, SOD, GSH-Px and CAT are the first-line antioxidant defence markers against the harmful effect of ROS by neutralization of the toxicity of oxidative damage. The results of various clinical evidence indicated that SOD, GSH-Px and CAT in hippocampus and plasma were increased in patients with AD [35]. Besides, some reports suggest that antioxidants may be recognized as a potential treatment for AD [29]. Our results showed that the activities of SOD, GSH-Px and CAT were decreased and the MDA content was increased in the hippocampus in AD rats. However, those rats treated by HCM showed a considerable increase in the activities of SOD, GSH-Px and CAT and a reduction in MDA content of hippocampus in a dose-related manner when compared to AD rats, indicating that HCM had a remarkable potential on strengthening antioxidant capacity of the body by enhancing the activity of endogenous antioxidant enzymes against oxidative stress and free radical side effects. The antioxidant effect of HCM is related to its ability to protect the neurons in hippocampal tissue.

We further investigated the expression level of Aβ1–42 in hippocampus tissue through immunohistochemistry and Western blotting. Our results showed that in the hippocampus of AD rats, the expression of Aβ1–42 was higher than that in control rats with serious impairment on learning and memory abilities. However, the spatial learning and memory abilities in the HCM group were notably enhanced, and the expression of Aβ1–42 in the hippocampus was decreased. We may infer that HCM could downregulate the expression of Aβ1–42 in the hippocampus, reduce the quantity of Aβ1–42 and thereby enable a neuroprotective and anti-oxidative stress effect in hippocampus tissue of AD rats.

In conclusion, our study successfully established the Aβ1–42 induced rat model and evaluated the beneficial effects of HCM on treatment of AD. Our findings suggested that intracerebroventricular injection of Aβ1–42 caused cognitive deficits and oxidative damage due to dysfunction of the antioxidant defence systems and overproduction of free radicals, as well as neuronal loss in the hippocampus tissue of AD rats. HCM treatment could decrease the expression of Aβ1–42 in hippocampus, and had a significant effect of enhancing the cognitive function and endogenous antioxidant status of Aβ1–42 induced AD rats through attenuating oxidative stress and neuroprotection effect. Further investigations into the molecular mechanisms should be engaged to substantiate its beneficial effects to AD and whether it can be an alternative remedy for neurodegenerative disease in the future.

Ethics approval

All experimental protocols were approved by the Laboratory Animal Centre of Medicine Animal Care and Use Committee.
Disclosure

The authors report no conflict of interest.

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