Effect of Donepezil Hydrochloride on Cognitive Function Recovery of Rats with Alzheimer’s Disease

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ABSTRACT

Objective: To explore the effect of donepezil hydrochloride on the cognitive function recovery of the rats with Alzheimer’s disease and the positive expression of nitric oxide synthase and acetylcholinesterase (NOS and AchE) in brain tissues of rats with Alzheimer’s disease (AD).

Methods: Forty SD (Sprague Dawley) rats were randomly classified into model group, treatment group, control group and sham operation group, with 10 rats in each group. AD rat models were prepared with A\textbeta\textsubscript{1-40}. The donepezil hydrochloride was orally administered to rats in treatment group two weeks after the modeling. Recognizing function of rats was assessed. The positive expression of NOS and AchE in brain tissues of each rat was observed via immunohistochemistry after administration of 28 days.

Results: The escape latency of rats was significantly longer after modeling ($p < 0.010$). Compared to the model group, the escape latency of rats in treatment group was significantly shortened and the difference was significant ($p < 0.05$). Positive expression of NOS and AchE is mainly reflected in hippocampus region in brain tissues of rats. Compared with control group and sham operation group, NOS positive expression decreased and AchE positive expression increased in the rat brain of model group. The difference was statistically significant ($p < 0.01$). Compared with model group, NOS expression in rat brain of treatment group increased and AchE expression decreased, and the different was significant ($p < 0.01$).

Conclusion: Donepezil hydrochloride can promote the recovery of cognitive function of AD rats. Its effect is also associated with the increased protein expression of NOS in hippocampus neuron cells in addition to specifically inhibiting the activities of AchE.

Key Words: Donepezil hydrochloride; Rats; Alzheimer’s disease
Alzheimer’s disease (AD) is a degenerative neurosis characterized mainly by cognitive and memorial dysfunctions, which is a common dementia among the elderly. With the coming of an aging society of population, AD has become the fourth disease that causes death after tumor, heart and cerebrovascular diseases. It has not only seriously reduced the quality of life of patients, but also brought a heavy burden to their families and the society. It has made clear that the learning and memory impairment of AD patients is associated with the brain cholinergic dysfunction. Donepezil hydrochloride belongs to a selective inhibitor of cholinesterase. A large number of clinical studies proved that it could significantly improve the cognitive and memorial function of AD patients and it was considered as an effective drug to treat the AD patients [1-2]. Therefore, it is of an important significance to positively explore the possible functional mechanism of donepezil hydrochloride except to inhibit the cholinesterase. In this study, AD model rats were taken as the research subject to study the effect of donepezil hydrochloride on NOS protein expression in hippocampal neurons and its effect on the AchE positive expression in the brain tissues of AD model rats. The results are reported as follows.

MATERIALS

1. Experimental Animals
40 SD male rats, weighed 200 ± 20 g, and provided by Hunan SJA Laboratory Animal Co., Ltd, with license number: SCXK (Hunan) 2009-0004.

2. Experimental Drug
Donepezil hydrochloride tablets (Shaanxi Ark Pharmaceutical Co., Ltd, national medicine permission number: H20030583). As per daily dosage (5 mg) for adult of 60 kg, the equivalent dosage for rats was 0.5 mg/kg. It was prepared into the suspension of 0.05 mg/mL with the distilled water.

3. Reagents and Instrument
amyloid β-protein 1-40(Aβ1-40), NOS and AchE polyclonal antibodies were purchased from Beijing Bios Biotechnology Co., Ltd; PV9000 kit and DAB chromogenic agent were purchased from Beijing ZSGB Biotechnology Co., Ltd; Morris water maze and brain stereotaxic apparatus (Shenzhen RWD Life Science Co., Ltd); Finesse 325 paraffin slicing machine (British Shando Corporation); Bx51 optical microscope and IPP6.0 image analysis system (Japanese Olympus Corporation).

METHODS

1. Grouping and Administration of Animals
All rats were fed for one week. They were then grouped into control group, sham operation group, model group and treatment group (model + Donepezil hydrochloride), with 10 rats in each group. The rats in treatment group were administered orally with donepezil hydrochloride suspension of 0.5mg/kg two weeks after the modeling, and the rats in sham operation group and model group were administered with normal saline. The rats in control group were fed normally. They were administered once a day, and this action continued 28 days.

2. Preparation of Model
AD rat model was prepared with Aβ1-40 in References [3]. Rat was anesthetized with 10 % chloral hydrate (3mL/kg) and fixed on the stereotaxic apparatus. A scalpel was used to cut an incision of 1.5cm long along the anterior median line in the disinfected area, find the bregma and adjust and locate it with the stereotaxic apparatus to ensure the syringe needle to be aligned to 2 mm at right side of the bregma and 3.5 m behind the bregma, which is the drilling point. Drill and open the skull, slowly push the syringe needle at a speed of 0.3 mm/min to 2.7 mm under the skull, which is exactly the hippocampus. Aβ1-40 was diluted into 5 ug/uL with sterile normal saline and cultivated at 37 °C to make it to become the aggregated state. Aβ1-40 of 5 ug/uL was slowly injected to the rat with a microinjector of 1uL at the injection speed of 0.1 uL/ min or so. After completing the injection, take out the needle slowly at a speed of 0.3 mm/min and seal the skull with dental mud and suture the skin. After completing the operation, rat was subject to a conventional feeding and intramuscular injection of penicillin to resist against the infection. 1uL of normal saline was injected into the hippocampus of rat in sham operation group and the operation method was identical to that in model group.

3. Evaluation of Learning and Memory in Rats
Morris water maze was used to test the learning and memory abilities of rats. In one week after modeling and three weeks after the drug administration, rats were subject to a water maze exercise of 7 days, once in morning and afternoon, respectively. On day
7, a place of entry was selected to put the rats into the water facing the pond wall to observe and record the time required for rats to find and climb onto the platform, namely, escape latency.

4. Test with NOS and AchE Immunohistochemistry

After completing the administration, all rats were anesthetized with chloral hydrate and killed to obtain their brains. They were fixed in 4 % paraformaldehyde for 24 hours. Brain tissues were dehydrated with gradient alcohol, hyalinized with xylene, infiltrated and embedded by paraffin, and sliced into coronal sections with thickness of 5 um using the slicing machine. Sections were put into 3 % hydrogen peroxide for incubation for 10 min, and washed for three times with 0.0 M PBS. They were then transferred to a microwave repair vat to be heated to boiling for 30 s, and then they were washed for three times with 0.01 M PBS. The sections of brain tissues of rat in various groups were dropped with 50 uL of diluted rabbit anti-rat NOS polyclonal antibody (1:100) and rabbit anti-rat AchE polyclonal antibody (1:100), respectively. The primary antibody in rats in negative control was replaced by 0.01M PBS. They were incubated in the refrigerator for 2h at 37 °C and washed with 0.01M PBS for three times. They were dropped with the goat anti-rabbit of PV9000 kit biotin labeled goat anti-rabbit, incubated for 30 min at 37 °C and washed with 0.01M PBS for three times. They were dropped with PV 9000 kit horseradish peroxidase labeled streptavidin, incubated for 20 min at 37 °C  and washed with 0.01M PBS for three times. They were dropped with DAB developer to develop the color for 10 min at a room temperature and washed with distilled water to terminate the reaction. The sections were stained with hematoxylin stain for 10 min and washed in running water for 5 min, and then were subject to color separation for 10 s in 0.5 % hydrochloric acid alcohol separation solution and washed in running water for 2 min. The sections were hyalinized with xylene and gradient alcohol and sealed with neutral resin. Three sections (slices) were taken from each brain tissue for each indicator. Each section was placed under the microscope of 400 times to select randomly five views without repeated positive expressions. The positive cells of the same unit area were counted using IPP 6.0 image processing system.

5. Statistical Analysis

SPSS19.0 statistical software was used for data analysis. All data are expressed by x±s, and the single factor analysis of variance was used for comparison among groups. LSD method was used for homogeneity of variance, and Tambane’s T2 method was used for heterogeneity of variance. P < 0.05 indicates the statistically significant difference.

RESULTS

1. Learning and Memory Situation of Rats in Various Groups.

The tests of escape latency of rats via the Morris water maze show a significant extension of escape latency of rats in model group and treatment group before the administration, and the difference is statistically significant compared with the result of control group and sham operation group (p < 0.01). Compared to the model group, the escape latency of rats in treatment group is significantly shortened after the administration (p < 0.05). See Table 1.

Table 1 Comparison of Escape Latency of Rats in Various Groups before and after Administration (x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Before Administration</th>
<th>After Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>10</td>
<td>38.26 ± 6.33</td>
<td>37.18 ± 5.92</td>
</tr>
<tr>
<td>Sham Operation</td>
<td>10</td>
<td>37.57 ± 7.02</td>
<td>36.50 ± 6.09</td>
</tr>
<tr>
<td>Model Group</td>
<td>10</td>
<td>92.65 ± 16.43</td>
<td>65.29 ± 13.71</td>
</tr>
<tr>
<td>Treatment Group</td>
<td>10</td>
<td>90.72 ± 17.80</td>
<td>44.04 ± 10.22</td>
</tr>
</tbody>
</table>

Note: 1. Compared with control group and sham operation group, p < 0.01; 2. Compared with model group, p < 0.05.

2. NOS and AchE Positive Expression in Rat Brain of Various Groups

The positive expression of NOS and AchE in brains of rats in various groups was detected by immunohistochemistry. The chocolate brown or brownish yellow stained in neuronal cell cytoplasm or protrusions present the positive expression. The main expression area is in hippocampus. The positive cells of the same unit area were counted using IPP 6.0 image processing system.
group ($p < 0.01$). After the administration of drugs, NOS expression in treatment group increased and AchE expression decreased, which indicates a statistically significant difference compared with the model group ($p < 0.01$), see Table 2 and Fig. 1 and 2.

### Table 2 NOS and AchE Positive Expressions in Rat Brains of Various Groups (x±s, piece/mm²)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>NOS</th>
<th>AchE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>10</td>
<td>116.85 ± 21.43$^{1)}$</td>
<td>96.36 ± 19.27$^{1)}$</td>
</tr>
<tr>
<td>Sham Operation Group</td>
<td>10</td>
<td>107.83 ± 19.61$^{1)}$</td>
<td>103.52 ± 24.85$^{1)}$</td>
</tr>
<tr>
<td>Model Group</td>
<td>10</td>
<td>59.52 ± 13.09</td>
<td>177.50 ± 35.43</td>
</tr>
<tr>
<td>Treatment Group</td>
<td>10</td>
<td>95.73 ± 17.30$^{1)}$</td>
<td>112.02 ± 22.35$^{1)}$</td>
</tr>
</tbody>
</table>

Notes: 1. Compared with model group, $P < 0.01$;

Discussion
So far, the pathogenesis of AD is still unclear. There are many theories concerning the etiology of AD, such as, genetics theory, β-amyloid protein (Aβ) deposition theory, cholinergic theory, inflammatory theory, excitatory amino acid theory, free radical damage theory and aluminum poisoning theory, etc. The pathology of AD disease is characterized by cerebral cortical atrophy, accompanied by Aβ deposition, neurofibrillary tangles, decreased memory neurons in a large number and the formation of senile plagues. At present, Aβ is generally accepted as the main factor in the induction of AD. The key for formation and development of the neuropathological process of AD is that Aβ sustains injury to the brain cells to lead to the progressive mental decline in patients and thus cause the formation of senile plagues. The Aβ deposition can decrease the choline acetyltransferase activities and cause the reduction of cholinergic neurons around Aβ deposition, thus leading to the memory impairment. It was reported that some drug can result in regression of Leydig cells and regressive and/or degenerative changes in the epididymis, seminal vesicle, ventral prostate and coagulating gland in male rats [4]. In this experiment, the current accepted method of acute injection of Aβ in the rat brain was used. It can cause the functional loss of cholinergic neurons and the dysfunction of learning and memory abilities, and result in the Aβ deposition in the brain. Therefore, it is a better animal model. In this experiment, the
escape latency of model rats extended two weeks after modeling (p < 0.01), indicating the decline of learning and memory ability of rats after modeling and a successful modeling.

The current accepted theory owes the cognitive symptoms of AD to a shortage of cholinergic neurotransmitters. Acetylcholine (Ach) is the important neurotransmitter in the cholinergic pathway of central nervous system, which has widely involved in the cognitive process of in-vivo learning and memory. The learning and memory ability decreases with the decrease of the Ach level. Donepezil hydrochloride is a hexaoxypyridine oxide and the generation-II specific reversible central AchE inhibitor. It can increase the concentration of Ach by inhibiting the hydrolysis of Ach to improve the cognitive function of AD patients [5]. However, Ach is not stable in the body and it can be hydrolyzed into choline and acetic acid. So, the activity level of AchE can indirectly reflect the Ach level. Some studies of animal experiments also reported that donepezil hydrochloride had the treatment effect on Aβ-induced AD rat [6] and it could significantly improve the learning and memory impairment of AD model rats and reduce the injury of free radicals to hippocampus neurons [7]. The results of this study also showed that the donepezil hydrochloride could improve the learning and memory function of AD rats (p < 0.05), indicating that it could inhibit the AchE activities in brains of rats and the AchE positive expression in hippocampal neuron cells of AD rats, and it can also be associated with the enhancement of NOS activity in brain hippocampus and the adjustment of the metabolism of messenger molecular NO in central nervous system in addition to the inhibition of the AchE activities.

**CONFLICT OF INTEREST STATEMENT**

No conflict of interests and financial disclosure is present. Qiliang Zhou contributes the manuscript, Siping Nie provides the technical guide and Zhihuo Liu provides the language editing service.

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