Expression of APP, BACE1, AChE and ChAT in an AD model in rats and the effect of donepezil hydrochloride treatment

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Abstract. The aim of this study was to investigate the pathological changes in a rat model of Alzheimer’s disease (AD) and the effect of donepezil hydrochloride (HCl) treatment. The rat model of AD was established by the bilateral injection of amyloid β1-40 (Aβ1-40) into the hippocampus. Changes in spatial learning and memory functions were examined using the Morris water maze test and changes in catalase (CAT) and glutathione peroxidase (GSH-Px) activities were determined using chemical colorimetry. Moreover, the changes in acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) expression were analyzed using immunohistochemical staining. The mRNA expression levels of the amyloid precursor protein (APP) and β-secreted enzyme 1 (BACE1) were evaluated using RT-PCR. The effects of donepezil HCl on the aforementioned indices were also observed. The rat memories of the platform quadrants in the blank, sham and donepezil HCl groups were improved compared with those of the rats in the model group. The ratio of swim distance in the fourth platform quadrant (l4/l total) was significantly decreased compared with that for the blank and sham group rats. Following donepezil HCl treatment, the ratio of l4/l total significantly increased. AD modeling caused a significant decrease in the CAT and GSH-Px activities in the brain tissues of the rats. The CAT and GSH-Px activities in the AD model rats significantly increased following donepezil HCl treatment. Moreover, donepezil HCl treatment significantly decreased the AChE, APP and BACE1 mRNA expression levels and increased the ChAT expression levels. Therefore, donepezil HCl was able to significantly decrease learning and memory damage in a rat model of AD.

Introduction

Alzheimer’s disease (AD) is a progressive degenerative disease of the central nervous system with clinical and pathological characteristics (1). In the brains of AD patients, amyloid deposits, whether in senile plaques or neurofibrillary tangles or in parts of the vascular walls of the brain, become β-amyloid proteins (Aβ) (2). A previous study has confirmed that mutation of the amyloid precursor protein (APP) gene may cause abnormalities in APP metabolism and Aβ deposition in the brain (3). APP gene mutation is found in 1 in 4 familial AD patients (4). Esh et al used a mutated APP gene (Val717-Phe717) to create a transgenic mouse model, which significantly expresses 717-mutated APP and gradually forms the neuropathological features of AD, including the appearance of β-amyloid plaques (5).

The superoxide radical produced by the metabolic process of aerobic cells has harmful effects, including biological damage and senility. The oxygen radical promotes the accumulation of Aβ and induces hyperactivity in the brain, causing neurodegeneration and, hence, AD (6). Previous studies have identified that the body’s ability to scavenge free radicals decreases with increasing age. Free radicals accumulate in the body and damage tissues and cells to accelerate the aging process (7) and are counteracted through a series of antioxidants, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT). When the normal defensive function declines, oxidative damage occurs and free radicals damage neurons and other cells (8).

Numerous studies have confirmed that AD accompanies various neurotransmitter disorders and that the acetylcholine (ACh) system is more affirmative in relation to AD (9). The central ACh neurotransmitter has important regulatory functions in learning and memory behavior. The maintenance of the normal functioning of this regulatory function is essential to ensure normal learning and memory (10). Furthermore, functional deficiency of the cholinergic system is closely related to AD, as confirmed by a number of autopsies and as accepted by most researchers. Numerous reports have shown that the expression of ACh and the metabolic enzyme, choline acetyltransferase (ChAT), is significantly decreased in the cerebral cortex and hippocampus in AD patients. The numbers of N receptors in the cerebral cortex and M receptors...
in the neuron-damaged area are also decreased, implying that AD is related to the loss of neurons in the nucleus basalis of Meynert (11). Certain studies have revealed that the degree of ACh decrease positively correlates with the severity of dementia (12).

Materials and methods

Animals and grouping. A total of 80 healthy male Sprague-Dawley (SD) rats were purchased from Beijing Weitong Lihua Test Animal Technology Co., Ltd (Beijing, China). Their weights were 280±10 g. They were randomly divided into four groups, namely, the blank, sham, model and donepezil hydrochloride (HCl) treatment groups. This study was approved by the Animal Care and Use Committee of Qiqihar Medical University.

Preparation of animal models (13). Following narcotization with 1% pentobarbital sodium (40 mg/kg), the rats were fixed on a stereotaxic apparatus. The skins on their cranial bones were preserved and the skin around the surgery area was routinely disinfected. A 2-3 cm incision was made along the cranial midline and the periosteum was detached. Then, a '+'-shaped suture and a lambdoid suture 3.0 mm behind the bregma were exposed and two 2.2 mm side openings were made around the central line. The skull was drilled using a dental auger and a microscale injector was inserted at 2.8 mm for the 1 µl solution injection. The injection time was 5 min and the needle was retained for 5 min to guarantee the diffusion of the solution. The needle was then pulled out slowly and the opening created in the skull was blocked with dental mud. The skin was disinfected with penicillin powder and then sutured. The other side of the hippocampus was injected following the same method. The model and donepezil HCl groups were injected with 10 µg µl Aβ1-40. The sham group was injected with normal saline and no treatment was administered to the blank group. Finally, 0.33 mg/kg/day donepezil HCl was injected into the donepezil HCl group through the stomach. Normal saline of a similar volume to that of the model group was injected into the donepezil HCl group to maintain the physiological condition. The rats were sacrificed by decapitation and the brains were removed and quickly frozen in liquid nitrogen and then prepared for RNA extraction. A total of nine rats from each group were sacrificed by decapitation and the brains were removed and quickly frozen. The left brains were weighed and prepared as brain homogenates. The right brains of six rats were fixed in a timely manner using 4% paraformaldehyde solution for light microscopic observation (immunohistochemistry).

Preparation of specimens. Following the behavioral testing, five rats from each group were sacrificed by decapitation and the brains were removed and quickly frozen in liquid nitrogen and then prepared for RNA extraction. A total of nine rats from each group were sacrificed by decapitation and the brains were removed and quickly frozen. The left brains were weighed and prepared as brain homogenates. The right brains of six rats were fixed in a timely manner using 4% paraformaldehyde solution for light microscopic observation (immunohistochemistry).

Determination of GSH-Px and CAT. The determination of the GSH-Px, CAT and protein contents of the brain homogenates was performed according to the instructions of the GSH-Px and CAT assay kit (Jiancheng Bioengineering, Nanjing, China).

Immunohistochemistry. The brain tissue obtained was sliced according to the paraffin method to a thickness of 5 µm. The wax was removed from the slice to allow hydration and the tissues were incubated with 3% H2O2 for 5 min to remove the endogenous CAT activity. The first antibody (dilution ratio of 1:200) was introduced and remained in contact with the tissue overnight at 4°C and the sample was then washed thrice with PBS liquid for 2 min. The second antibody (goat anti-rabbit) working solution was then added to the sample which was maintained at room temperature for 20 min. The sample was then washed thrice with PBS liquid for 2 min. The DAB coloration was observed under a light microscope. The sample was washed with distilled water to terminate the chromogenic reaction, redyed using hematoxylin for 2 min and gradually dehydrated with alcohol (80, 90, 95 and 100%) for 2 min for each alcohol concentration. The tissues were rendered transparent with dimethylbenzene and fixation was tested on a slide with resin.

RT-PCR. The total brain RNA was extracted using a TRizol kit (Invitrogen, Carlsbad, CA, USA). A total of 100 mg rat brain tissue was placed into a glass homogenizer. The total RNA extraction was performed according to the kit's instructions provided by the manufacturer. First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen, Shanghai, China). The reverse transcription reaction was performed according to the kit's instructions and synthetic cDNA was used to create the polymerase chain reaction. The primer sequences were as follows: the forward and reverse sequences for APP were 5'-TGGGTTGACAAACCATCAAGACAGAA-3' and 5'-GCACCTTTGTGTGAACCCACATCT-3', respectively, with 135 bp produced. Those of the β-secreted enzyme 1 (BACE1) were 5'-TGTTGAGACACGGGCAGTAGTA-3' and 5'-TCGGAGGTCTCGGTATGTACTGG-3', respectively, with 104 bp produced. Finally, those for phosphoglyceraldehyde dehydrogenase (GAPDH) were 5'-GACAACCTTTGCGCATC GTGGA-3' and 5'-ATGCAGGGATGTGTTCTGG-3', respectively, with 104 bp produced. The primers were designed using Primer 5.0.
Table I. Learning and memory ability of AD rats and the effects of donepezil hydrochloride on them (mean ± SD).

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>I₁</th>
<th>I₂</th>
<th>I₃</th>
<th>I₄</th>
<th>I₁/I total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>20</td>
<td>323.89±40.58</td>
<td>391.35±38.56</td>
<td>334.90±49.38</td>
<td>758.01±72.14</td>
<td>41.95±3.65</td>
</tr>
<tr>
<td>Sham</td>
<td>19</td>
<td>312.12±48.91</td>
<td>402.52±42.35</td>
<td>339.88±53.47</td>
<td>750.99±70.11</td>
<td>41.64±3.27</td>
</tr>
<tr>
<td>Model</td>
<td>18</td>
<td>346.57±50.65</td>
<td>411.18±44.17</td>
<td>443.31±48.70</td>
<td>389.78±58.34</td>
<td>24.44±2.76</td>
</tr>
<tr>
<td>Donepezil HCl</td>
<td>19</td>
<td>317.99±37.45</td>
<td>396.44±49.54</td>
<td>501.11±44.39</td>
<td>721.01±45.20</td>
<td>37.28±2.42</td>
</tr>
</tbody>
</table>

AD, Alzheimer's disease; HCl, hydrochloride. Compared with other quadrants, *P<0.05, †P<0.01. ‡Compared with the sham groups, P<0.01.

Table II. Levels of GSH-Px and CAT in AD rat brain tissue and the effects of donepezil hydrochloride on them (mean ± SD).

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>CAT (U/mg x protein)</th>
<th>GSH-Px (U/mg x protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>9</td>
<td>17.58±1.59</td>
<td>173.59±16.26</td>
</tr>
<tr>
<td>Sham</td>
<td>9</td>
<td>16.55±1.29</td>
<td>164.60±14.70</td>
</tr>
<tr>
<td>Model</td>
<td>9</td>
<td>11.05±1.49*a</td>
<td>123.34±14.02*a</td>
</tr>
<tr>
<td>Donepezil HCl</td>
<td>9</td>
<td>13.10±1.40*b</td>
<td>141.44±13.12*b</td>
</tr>
</tbody>
</table>

GSH-Px, glutathione peroxidase; CAT, catalase; AD, Alzheimer’s disease; HCl, hydrochloride. *Compared with the blank and sham groups, P<0.01; †Compared with the model group P<0.01; ‡Compared with the sham, P<0.01.

Table III. Levels of AChE and ChAT in AD rat brain and the effects of donepezil hydrochloride on them (mean ± SD).

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>AChE</th>
<th>ChAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>6</td>
<td>0.086±0.011</td>
<td>0.115±0.024</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>0.079±0.010</td>
<td>0.094±0.026</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>0.045±0.008*a</td>
<td>0.046±0.012*a</td>
</tr>
<tr>
<td>Donepezil HCl</td>
<td>6</td>
<td>0.065±0.014*c</td>
<td>0.072±0.014*b</td>
</tr>
</tbody>
</table>

AChE, acetylcholinesterase; ChAT, choline acetyltransferase; AD, Alzheimer’s disease; HCl, hydrochloride. *Compared with the blank and sham groups, P<0.01; †Compared with the model group P<0.01; ‡Compared with the sham, P<0.01.

Results

Effects of donepezil HCl on learning and memory ability in a rat model of AD. As shown in Table I, the swim distance of the fourth quadrant was significantly different (P<0.05 or <0.01) from those of the other quadrants in the blank, sham and donepezil HCl groups. No significant differences were identified in the swim distance for each quadrant in the model group. These results indicate that the rat memories of the platform quadrant in the blank, sham and donepezil HCl groups improved compared with those of the model group rats. The ratios of the swim distance in the fourth platform quadrant (I₄) to the total swim distance (I total) of each group (I₄/I total) were calculated. The I₄/I total of the model group was significantly lower than that of the blank and sham groups (P<0.01). However, the I₄/I total ratio of the donepezil HCl group was significantly increased compared with that of the model group (P<0.01).

Acetylcholinesterase (AChE) and ChAT expression. As shown in Table III, no significant differences were found between the AChE and ChAT levels in the rats of the blank and sham groups (P>0.05). The GSH-Px and CAT levels in the rat brains significantly decreased following AD modeling compared with those of the blank and sham groups (P<0.01). Moreover, the GSH-Px and CAT activities in the AD rat brains significantly increased following donepezil HCl treatment compared with those in the rat brains of the model group (P<0.05 or <0.01).
Alzheimer’s disease; HCl, acetylcholine esterase. A number of studies have shown increases in acetylcholinesterase activity are observed in the brains of AD patients (15). Thus, learning and memory are important factors in the diagnosis of AD and for the evaluation of its treatment (16).

In the present experimental study, the Morris water maze system was used to examine the learning and memory abilities of each group of rats. The swim trajectories of the normal and approximately normal rats were predominantly scattered in the fourth platform quadrant. The memory-deficient rats aimlessly searched for a means of escape, and thus, their swim trajectories were chaotic. Moreover, the distance of aimless swim significantly increased as the spatial location memory of the rats decreased and they could not remember the location of the platform. The results showed that the swim distance in the fourth quadrant for the model group was significantly different from that in the other quadrants for the blank, sham and donepezil HCl groups (P<0.05 or <0.01).

The increased production of free radicals in AD patients is one of the causes of the changes in brain structure and function (17). Studies have shown that free radicals may promote the accumulation of Aβ and induce hyperactivity in the brain, causing neurodegeneration and, hence, AD. The increase in oxygen radicals may result in tau protein abnormal phosphorylation and neurofibrillar tangles, therefore causing functional degradation of the nerve cell, or even death, and aggravating the course of AD (18). Other studies have shown that free radicals may induce cell apoptosis and cause AD. The defensive reaction of cells against free radicals is of two types. The first is the defensive reaction of enzymes, including SOD, CAT and GSH-Px. SOD converts superoxide free radicals into H₂O₂. The low concentration of H₂O₂ is converted into water and molecular oxygen. GSH is used as proton donor and the high concentration of H₂O₂ is removed by CAT (19). CAT and GSH-Px are significant enzymes in the removal of free radicals as they transform the H₂O₂ produced by SOD, catalyzing the disproportionation of two superoxide radicals into water (20). The present experiment shows that the AD modeling by bilateral Aβ₁₋₄₀ injection into the hippocampus of rats may inhibit the free radical defensive function and lead to oxidative damage. Donepezil HCl significantly increases the levels of GSH-Px and CAT. The antioxidase activities of CAT or GSH-Px may be adjusted for indirect antioxidation.

ACh participates in the regulation of the neuronal activity of the hippocampus and neocortex, it is widely spread along the synaptic cleft of the cholinergic synapse and is neurotransmitted for the promotion of learning and memory. ACh is also an important index that reflects cholinergic nerve function and is significant in the memory and cognition damage of AD patients (21). ACh is synthesized by the ChAT-catalyzed reaction of choline with acetyl-CoA and resolved by AChE. ChAT and AChE together maintain the dynamic balance of ACh (22). AChE has high catalytic activity and inactivates ACh by resolving it into choline and acetic acid to ensure effective transfer at the cholinergic nerve. The activity of AChE directly reflects the functional state of the cholinergic system.

Cholinesterase inhibitors have been used clinically to treat AD. Inhibiting the activity of cholinesterase may increase the ACh content of the brain and lead to a recovery of cholinergic nerve transmission. Cholinesterase inhibitors have achieved beneficial treatment effects in mild and moderate AD patients. ChAT is the rate-limiting enzyme of ACh synthesis, a focus of studies of cholinergic neurons and an indirect evaluation index for the levels of ACh that are released. A number of studies have shown decreases in the activity of ChAT of 49-90% in the cerebral cortex, hippocampus and basal nucleus of telencephalon group of AD patients. Moreover, the degree of the decrease in activity is closely related to the degree of dementia (23). ACh is very unstable and difficult to determine due to its rapid rate of hydrolysis. Therefore, the functioning

| Table IV. mRNA expression of APP in AD rat brain tissue and the effect of donepezil hydrochloride on it (mean ± SD). |
|---|---|---|
| Groups | No. | Mean ± SD |
| Blank | 5 | 0.82±0.21 |
| Sham | 5 | 0.92±0.21 |
| Model | 5 | 1.74±0.23* |
| Donepezil HCl | 5 | 1.14±0.19* |

APP, amyloid precursor protein; AD, Alzheimer's disease; HCl, hydrochloride. ´Compared with the blank and sham groups, P<0.01. *Compared with the model group, P<0.01.

| Table V. mRNA expression of BACE1 in AD rat brain tissue and the effect of donepezil hydrochloride on it (mean ± SD). |
|---|---|---|
| Groups | No. | Mean ± SD |
| Blank | 5 | 0.54±0.14 |
| Sham | 5 | 0.61±0.11 |
| Model | 5 | 1.12±0.34* |
| Donepezil HCl | 5 | 0.74±0.17* |

BACE1, β-secreted enzyme 1; AD, Alzheimer's disease. ´Compared with the blank and sham groups, P<0.01. *Compared with the model group, P<0.05.

**APP and BACE1 mRNA expression.** The results in Table IV show no significant difference between APP levels in the rats of the blank and sham groups (P>0.05). The APP mRNA expression levels in rats of the model group were significantly increased compared with those in rats of the blank and sham groups (P<0.01), whereas those in the donepezil HCl group rats were significantly decreased compared with those in the model group rats (P<0.01).

Moreover, the results in Table V show no significant difference between the BACE1 levels in the rats of the blank and sham groups (P>0.05). The BACE1 mRNA expression levels in the rats of the model group were significantly increased compared with those in the rats of the blank and sham groups (P<0.01; Table V), whereas those of the donepezil HCl group rats were significantly decreased compared with those of the model group rats (P<0.05).

**Discussion**

The clinical manifestations of AD include hypophrenia, recent memory loss and obstacles in the related capacity for action (15). Thus, learning and memory are important factors in AD diagnosis and for the evaluation of its treatment (16). In the present experimental study, the Morris water maze system was used to examine the learning and memory abilities of each group of rats. The swim trajectories of the normal and approximately normal rats were predominantly scattered in the fourth platform quadrant. The memory-deficient rats...
of the cholinergic system is usually observed through the activities of AChE and ChAT. The present experimental results show that in the AD model group of rats the AChE expression increases and the ChAT expression decreased.

Aβ production is the initial step in SP formation and very high concentrations of Aβ may be identified in brains of AD patients. Aβ is toxic to neurons and causes the denaturation and death of neuronal cells (24). Moreover, Aβ may worsen the damaging effects of free radicals, increase the inflammatory response to cell factor, disturb ionic equilibrium and induce cell apoptosis (25). The Aβ pathway is common to AD of various causes and is central to the pathological mechanism of AD. Aβ has been confirmed to be generated from the gene that codes APP (26). Jeong et al found that the mRNA levels of β-APP and APP in the brain hippocampus increased with age when they tested senile aged mice (SAM). Moreover, they stated that the excessive expression of hippocampal APP was related to the memory loss of the SAM (27). Therefore, the decrease in the APP mRNA expression may inhibit Aβ accumulation in the brain. The APP protein processing paths have predominantly amyloid and non-amyloid peptide sources. β and γ secretases together induce APP to produce the pathological Aβ (28). β-secretase is a pivotal rate-limiting enzyme of the Aβ production process. The Aβ level may be regulated by the activation or suppression of β-secretase. Therefore, the use of β-secretase, also called BACE or BACE1, as a target is expected to become an ideal method of AD treatment (29).

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References


