ORIGINAL ARTICLE

# Effect and mechanism of Donepezil hydrochloride on Alzheimer's disease

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## ABSTRACT

**Objective:** To explore the effect and mechanism of donepezil hydrochloride on Alzheimer's disease (AD).
**Methods:** Thirty-six 3-month-old SD rats were selected as the research subjects and randomly divided into a normal control (NC) group and a model group. The model group was given continuous intraperitoneal injection of D-galactose solution (120 mg/kg/d) combined with aluminum trichloride (10 mg/kg/d) by gavage for 60 days. Morris water maze test was conducted to test the learning and memory abilities of the rats, and AD rats were selected. After modeling, AD rats were divided into a normal saline (NS) group and a drug treatment (DT) group. The DT group was given donepezil hydrochloride (1.0 mg/kg) by gavage intervention, and the NC group and the NS group were given equal volumes of physiological saline by gavage. After 4 weeks of intervention, Morris water maze test was conducted to detect the escape latency, the number of platform crossings and residence time in the target quadrant, the rats were euthanized, with serum and hippocampal tissues collected, and hippocampal tissue homogenate (10%) was prepared by using NS. Enzyme linked immunosorbent assay (ELISA) was used to detect the inflammatory and oxidative stress indicators in rat hippocampal tissues and serum, and hematoxylin eosin (HE) staining was used to observe pathological damage in rat hippocampal tissues.
**Results:** Compared with the NC group, the NS group showed a significant increase in escape latency ($p < .05$), a significant decrease in the number of platform crossings and residence time in the target quadrant ($p < .05$), and a significant decrease in the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in serum and hippocampal tissues ($p < .05$). The content of malondialdehyde (MDA) was significantly increased ($p < .05$), and the levels of interleukin-6 (IL-6) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) were significantly increased ($p < .05$). Compared with the NS group, the DT group can significantly reduce the escape latency ($p < .05$), increase the number of platform crossings and residence time in the target quadrant ($p < .05$), significantly increase the activities of SOD and GSH-Px in serum and hippocampal tissues and reduce the content of MDA ($p < .05$) and the levels of IL-6 and TNF-$\alpha$ ($p < .05$). After intervention with donepezil hydrochloride, the number of neurons in the hippocampus were significantly increased.
**Conclusions:** Donepezil hydrochloride can improve the learning and memory abilities of rats, reduce the levels of oxidative stress and inflammation in the brain and serum, and improve pathological damage in the hippocampus.

**Key Words:** Donepezil hydrochloride, Alzheimer's disease, Ability to learn and remember, Oxidative stress, Inflammation

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## 1. INTRODUCTION

Alzheimer's disease (AD), a degenerative disease of the central nervous system characterized by progressive cognitive dysfunction and behavioral impairment, is the most common form of dementia in elderly adults, with about 50 million people worldwide suffering from dementia, of which AD accounts for 60% to 70%.[1] This figure is expected to double in the next 20 years, reaching up to 150 million by mid-century,[2] placing a heavy burden on the society and families. At present, there is no treatment plan for AD, and only drugs and symptomatic treatment are used to delay the progress of cognitive decline.[3] Donepezil hydrochloride is one of the most commonly used acetylcholinesterase inhibitors in the treatment of AD, which increases cholinergic transmission by reversibly inhibiting the hydrolysis of acetylcholine by acetylcholinesterase, thereby improving patients' cognitive function and memory, and has the characteristics of high selectivity, reversibility and long duration of action. Donepezil hydrochloride can inhibit glutamate toxicity, activate N receptors, reduce the damage of free radicals to brain cells, and protect brain cells, which is a common drug for the treatment of mild to moderate AD. At present, the clinical efficacy of donepezil hydrochloride in the treatment of AD varies greatly, and there are no clear studies to clarify the effect of donepezil hydrochloride on the level of oxidative stress and inflammation in the brain of AD rats. In this study, donepezil hydrochloride was used to intervene in AD rats to observe its effects on the levels of oxidative stress and inflammation in hippocampal tissue and serum, as well as pathological damage in hippocampal tissue.

## 2. MATERIALS

### 2.1 Experimental animals

Thirty-six healthy male SD rats (3 months old) without specific pathogens (SPF grade), weighing 300-400 g, were purchased from SPF (Beijing) Biotechnology Co., Ltd with license number SCXK (Beijing) 2019-0010. Before the experiment, all rats were placed in the barrier environment in Experimental Animal Center, the feeding environment was ventilated, with the constant temperature (temperature (22 $\pm$ 2) °C) and humidity (50%-70%), the light was uniform, and the animals were given separate cage feeding, 5 in 1 cage. Each cage of rats had free access to food and water. and the experiment began after a week of adaptive feeding, and all rats were given humanitarian care during the experiment.

### 2.2 Experimental drugs and reagents

D-galactose was purchased from Sinopharm Chemical Reagent Co., Ltd; Aluminum trichloride was purchased from Shanghai Aibi Chemical Reagent Brand Store; dilute hydrochloric acid was purchased from Guangzhou Howei Pharma Tech Co.,Ltd; Donepezil hydrochloride tablets were purchased from Jiangsu Hansoh Pharmaceutical Group Co., Ltd.; Glutathione peroxidase, superoxide dismutase, malondialdehyde, interleukin-6 and tumor necrosis factor-$\alpha$ kit were purchased from Jiangsu Zeyu Biotechnology Co., Ltd.; The HE staining kit was purchased from Solarbio Corporation.

## 3. EXPERIMENTAL METHODS

### 3.1 Establishment and screening of AD rat models

After 1 week of adaptive feeding, 36 healthy male rats were divided into the normal control (NC) group and the model group according to the random number table method, including 6 in the NC group and 30 in the model group, and the model group was given continuous intraperitoneal injection of D-galactose solution (120 mg/kg/d) combined with aluminum trichloride (10 mg/kg/d) by gavage for 60 days, and the Morris water maze was performed to test the learning and memory abilities of the rats: Firstly, 6 rats in the NC group were tested by means of Morris water maze, continuously trained for 5 days, and placed in four different quadrants for training every day, each quadrant was tested once, and the test results were used as the reference standards for the screening of AD rats; Subsequently, 30 rats in the model group underwent Morris water maze behavior test, and the method was the same as that of the NC group. The test results of normal rats were used as the control, and the mean mean escape latency of normal rats was + 2 times the standard deviation as the lower limit, and the + 1 standard deviation value was used as the upper limit. The rats in the model group were divided into two groups: those with an average escape latency + 2 times greater than the standard deviation of the mean value were the successful AD rat models, and those with + 1 time less than the standard deviation of the mean value were the unsuccessful AD rat models. Rats with an incubation period between the upper and lower limits were excluded.

### 3.2 Grouping and method of administration

The AD rats were divided into the normal saline (NS) group and the drug treatment (DT) group, according to the random number table method, 6 rats in each group, and the DT group was given donepezil hydrochloride (1.0 mg/kg) for gavage intervention, and the NC group and the NS group were given the same volume of NS gavage for 4 weeks.

### 3.3 Detection of learning and memory abilities in rats by Morris water maze test

The Morris Water Maze is a circular tank with a radius of 80 cm and a height of 60 cm, filled with water at about 25°C. Ink is added to the water to make the water opaque, and the

tank is divided into 4 quadrants, the fourth of which contains a circular escape platform (about 12 cm in diameter), placed in a fixed position, 2.5 cm below the surface of the water, with visual cues around the water maze. The experiment was carried out 24 hours after the last administration, and the experimental content included the first 5 days of positioning navigation training and the $6th$ day of space exploration experiment, the positioning navigation test was carried out 4 times a day, once in each quadrant, and the training interval of each quadrant was 1 hour for 5 consecutive days. In each trial, the animal was placed in water in one of the four quadrants gently facing the pool wall, the starting quadrant varies randomly in the trial, the rat was required to find the escape platform within 90 seconds and stay on the escape platform for 3 seconds, and if the rat does not find the escape platform within 90 s (counted as 90 s), the rat was guided to find the escape platform and stay in the platform for 10 s. In all training trials, the time it took for the rat to reach the underwater platform (i.e., escape latency) was recorded to assess the rat ability to learn. On Day 6, another set of tests (i.e., the space exploration experiment) were conducted, in which the platform in the fourth quadrant was removed, each rat was lowered into the water from the second quadrant with the head towards the pool wall, the video tracking device collected the time the rats stayed in the fourth quadrant and the number of times of platform crossings, and the data were processed to assess the spatial exploration ability.

### 3.4 Detection of the activities of SOD and GSH-Px and the levels of MDA, TNF-$\alpha$ and IL-6 in rat serum by enzyme linked immunosorbent assay (ELISA)

After the completion of the Morris water maze test, the rats were fastened for 12 hours and then weighed. The intraperitoneal anesthesia with sodium pentobarbital (40 mg/kg) was applied, and abdominal aortic blood collection was carried out, followed by centrifugation in a centrifuge for 15 min (3,500 r/min), with the supernatant taken for later use. Inflammatory factors and oxidative stress indicators were detected in strict accordance with the instructions of the kit.

### 3.5 The detection of the activities of SOD and GSH-Px and the levels of MDA, TNF-$\alpha$ and IL-6 in rat hippocampus tissues by ELISA

After the completion of the Morris water maze test, the rats were fastened for 12 hours and then weighed. The intraperitoneal anesthesia with sodium pentobarbital (40 mg/kg) was applied, and then the rats were sacrificed by cervical dislocation. Whole brains were removed on ice, hippocampal tissues were quickly isolated, weighed separately and quickly placed in liquid nitrogen for snap-freezing, followed by the placement in a -80 °C freezer. The hippocampal tissues were crushed in an ice bath in a mortar to make a 10% hippocampal tissue homogenate, and centrifuged at 4 °C at a speed of 3,000 r/min for 10 min, with the supernatant retained. Inflammatory factors and oxidative stress indicators were detected in strict accordance with the instructions of the kit.

### 3.6 Pathological staining

The rat hippocampal tissues were fixed, after dehydration, wax immersion, embedding, section preparation, and then took out and stored at room temperature for later use, and the pathological changes of the sections were observed by microscopic examination according to the instruction for use of HE staining kit.

### 3.7 Statistical treatment

SPSS 26.0 statistical software was used to make a statistical analysis, and the measurement data were represented by mean $\pm$ standard deviation ($\bar{x} \pm$ s). Repeated measures ANOVA was used to process the latency data of Water maze test. The $t$-test was applied to the comparison between two groups and one-way ANOVA was applied to the comparison among groups. $p < .05$ was statistically significant.

## 4. RESULTS

### 4.1 Effect of donepezil hydrochloride on the behavioral-insidious escape latency of rats with AD

After the completion of drug intervention, the water maze test was carried out, and with the progress of training, it was found that the insidious escape latency of the NS group was significantly longer than that of the NC group, and the difference was statistically significant ($p < .05$). Compared with the NS group, the insidious escape latency in the DT group was significantly shortened, and the difference was statistically significant ($p < .05$). The results suggest that donepezil hydrochloride improved the learning ability of rats with AD (see Table 1).

### 4.2 Effect of donepezil hydrochloride on behavioral-space exploration experiments in rats with AD

After the completion of drug intervention, the water maze test was carried out until Day 6, the platform of the fourth quadrant was removed, the spatial exploration ability of the rats was tested, and the number of platform crossings and the time they stayed in the fourth quadrant were recorded. Compared with the NC group, the number of platform crossings and the time they stayed in the fourth quadrant were significantly reduced in the NS group, and the difference was statistically significant ($p < .05$). Compared with the NS group, the number of platform crossings and the time they stayed in the fourth quadrant were significantly increased in the DT group, and the difference was statistically significant

($p < .05$). The results showed that donepezil hydrochloride improved the memory ability of rats with AD. The results are shown in Table 2.

### 4.3 Effect of donepezil hydrochloride on the serum inflammation and oxidative stress indicators in rats with AD

Compared with the NC group, the levels of IL-6 and TNF-$\alpha$ in the NS group were significantly higher than those in the NC group, and the activities of SOD and GSH-Px were significantly reduced, and the content of MDA was significantly higher ($p < .05$). Compared with the NS group, the levels of IL-6 and TNF-$\alpha$ in the DT group were significantly decreased, the activities of SOD and GSH-Px were significantly increased, and the content of MDA was significantly decreased, the difference was of statistical significance ($p < .05$). The results showed that donepezil hydrochloride could reduce the levels of inflammation and oxidative stress in the serum of rats with AD (see Table 3).

### 4.4 Effect of donepezil hydrochloride on the hippocampal inflammation and oxidative stress indicators in rats with AD

Compared with the NC group, the levels of IL-6 and TNF-$\alpha$ in the hippocampus tissues were significantly increased, the activities of SOD and GSH-Px were significantly decreased, and the content of MDA was significantly increased in the NS group, and the difference was statistically significant ($p < .05$). Compared with the NS group, the levels of IL-6 and TNF-$\alpha$ of hippocampal tissues were significantly decreased, the activities of SOD and GSH-Px were significantly increased, and the content of MDA was significantly decreased in the DT group, and the difference was statistically significant ($p < .05$). The results showed that donepezil hydrochloride could reduce the levels of inflammation and oxidative stress indicators in the hippocampus tissues of rats with AD (see Table 4).

### 4.5 Effect of donepezil hydrochloride on histopathological damage of hippocampal tissues in rats with AD

Through HE staining, as shown in Figure 1, the pathological changes of the hippocampal tissues in the NC group were not obvious, the neuronal staining was uniform, the number of neurons was abundant, the arrangement was neat, the hierarchy was distinct, the nucleus was large and round, the chromatin was less, the nucleoli were obvious, and there was no obvious neuronal edema and necrosis. In the NS group, the number of neurons was significantly reduced, the neurons were arranged in an irregular manner, and there were phenomena such as nuclear condensation and cell body atrophy. The number of neurons in the DT group was higher than that in the NS group, and the neuronal arrangement was more neat than that in the NS group.

**Table 1.** Comparison of Morris water maze escape latency in each group of rats (n = 6)

| Group | Day 1 (S) | Day 2 (S) | Day 3 (S) | Day 4 (S) | Day 5 (S) |
|---|---|---|---|---|---|
| Normal control group | 56.07±3.25 | 43.68±3.16$^{\&}$ | 37.89±2.77$^{\&}$ | 23.80±3.16$^{\&}$ | 9.19±1.19$^{\&}$ |
| Normal saline group | 60.20±2.74$^{*}$ | 52.35±1.03$^{*\&}$ | 52.16±4.92$^{*\&}$ | 50.95±3.27$^{*\&}$ | 47.49±5.01$^{*\&}$ |
| Drug treatment group | 58.25±1.97$^{\#}$ | 49.14±1.02$^{*\#\&}$ | 44.10±2.74$^{*\#\&}$ | 37.86±1.57$^{*\#\&}$ | 27.19±3.05$^{*\#\&}$ |

*Note*. Compared with the normal control group, $^{*}p < .05$; Compared with the normal saline group, $^{\#}p < .05$; Compared with the previous day in the same group, $^{\&}p < .05$

**Table 2.** Comparison of the number of times rats crossed the platform and the residence time in the original platform quadrant on the $6^{th}$ day of Morris water maze in each group of rats ($\bar{x} \pm$ s, n = 6)

| Group | The number of platform crossings | Staying time in the original platform quadrant (s) |
|---|---|---|
| Normal control group | 4.67±1.21 | 28.68±7.66 |
| Normal saline group | 1.17±0.75$^{*}$ | 14.99±3.05$^{*}$ |
| Drug control group | 3.00±0.89$^{*\#}$ | 20.16±1.51$^{\#}$ |

*Note*. Compared with the normal control group, $^{*}p < .05$; Compared with the normal saline group, $^{\#}p < .05$

## 5. DISCUSSION

There are many causes of AD and the pathogenesis is not clear, and the main pathogenesis is abnormal deposition of $\beta$-amyloid protein (A$\beta$) and hyperphosphorylation of tau protein AD.[4] Studies have shown[5] that the deposition of $\beta$-amyloid (A$\beta$) triggers the generation of reactive oxygen free radicals, which in turn promote the increase of A$\beta$ deposition, leading to mitochondrial dysfunction and damage to neuronal cell membranes, and ultimately promoting the occurrence and development of AD. Studies have shown[6] that the combination of aluminum trichloride and D-galactose (D-gal) can cause neurotoxicity, which is a widely recognized and good AD model induction method at home and abroad. In this study, the cognitive function, learning and

memory ability of rats were detected by Morris water maze, and the improvement effect of donepezil hydrochloride on AD rats was observed, and the mechanism of donepezil hydrochloride was further explored from the perspectives of anti-inflammatory, antioxidative stress and hippocampal histopathological damage.

**Table 3.** Comparison of inflammatory and oxidative stress indicators in serum of rats in different groups ($\bar{x}\pm$ s, n = 6)

| Group | IL-6 (pg/ml) | TNF-α (pg/ml) | SOD (pg/ml) | GSH-px (U/ml) | MDA (nmol/ml) |
|---|---|---|---|---|---|
| Normal control group | 32.25±2.11 | 68.94±3.27 | 151.82±10.26 | 371.01±21.89 | 18.72±3.41 |
| Normal saline group | 41.49±1.29$^{*}$ | 91.73±4.46$^{*}$ | 105.03±8.68$^{*}$ | 252.78±22.23$^{*}$ | 33.02±2.04$^{*}$ |
| Drug control group | 38.51±1.52$^{*\#}$ | 80.18±4.27$^{*\#}$ | 129.10±9.63$^{*\#}$ | 308.55±18.25$^{*\#}$ | 27.13±1.84$^{*\#}$ |

*Note*. Compared with the normal control group, $^{*}p < .05$; Compared with the normal saline group, the $^{\#}p < .05$

**Table 4.** Comparison of inflammatory and oxidative stress indicators in hippocampal tissue of rats in different groups ($\bar{x}\pm$ s, n = 6)

| Group | IL-6 (pg/ml) | TNF-α (pg/ml) | SOD (pg/ml) | GSH-px (U/ml) | MDA (nmol/ml) |
|---|---|---|---|---|---|
| Normal control group | 29.35±2.56 | 73.81±4.60 | 146.53±6.08 | 411.93±26.45 | 3.08±0.67 |
| Normal saline group | 43.98±3.54$^{*}$ | 95.38±3.01$^{*}$ | 98.79±7.83$^{*}$ | 216.09±16.13$^{*}$ | 20.01±1.01$^{*}$ |
| Drug treatment group | 38.76±1.43$^{*\#}$ | 85.01±3.40$^{*\#}$ | 128.85±5.70$^{*\#}$ | 301.89±19.30$^{*\#}$ | 13.03±1.40$^{*\#}$ |

*Note*. Compared with the normal control group, $^{*}p < .05$; Compared with the normal saline group, the $^{\#}p < .05$

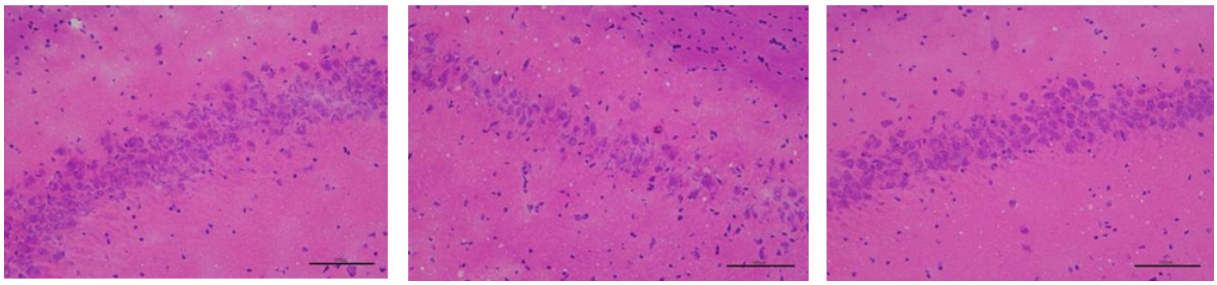

**Figure 1.** HE staining of hippocampal tissues in each group of rats (×200)

The Morris water maze test is often used to detect the cognitive ability of rodents and hippocampus-related spatial position and direction, and is also widely used in the study of learning and memory, animal psychology and behavior, and other disciplines. It is the first choice for animal behavior research, especially learning and memory research.[7,8] The results of the water maze test showed a significant increase in the latency of AD rats, which was consistent with previous studies,[9] indicating the success of the AD model. In this study, it was found that rats in the NS group took longer to find a platform than rats in the DT group, and AD rats in the NS group also crossed the platform and stayed in the target quadrant less often, which is consistent with the results of other studies.[10] These results indicated that donepezil hydrochloride alleviated D-galactose and AlCl3-induced memory dysfunction and delayed the development of AD.

Neuroinflammation is one of the main features of AD, and studies have shown that the activation of microglia helps to accelerate phagocytosis, degradation and clearance of A$\beta$, thereby avoiding the accumulation of A$\beta$ in the brain, but with the prolonged activation of immune cells, it will lead to the release of pro-inflammatory cytokines, triggering an inflammatory cascade, which will eventually lead to the deposition of A$\beta$ and hyperphosphorylation of Tau protein, which in turn accelerates or exacerbates neurodegenerative processes, and ultimately leads to cognitive decline.[11,12] TNF-$\alpha$ is involved in many systemic chronic inflammatory and degenerative diseases, is one of the key mediators of neuroinflammation, and plays a central role in the cytokine cascade of inflammatory responses, TNF-$\alpha$ can stimulate the generation of IL-6, IL-8 and other factors, so that the body can produce persistent inflammatory responses. The results of this study showed that the levels of IL-6 and TNF-$\alpha$ in serum and hippocampal tissues in the DT group were significantly lower than those in the NS group, suggesting that donepezil hydrochloride can reduce neuroinflammatory response by reducing pro-inflammatory factors, thereby improving patients' cognitive dysfunction. Oxidative stress is

an important mechanism in the early pathogenesis of AD, which not only participates in the generation of A$\beta$, but also participates in the phosphorylation of Tau proteins, and oxidative stress will promote the deposition of A$\beta$ and the hyperphosphorylation of Tau proteins, affect mitochondrial function, and cause synaptic dysfunction and even neuronal death.[13] Conversely, abnormal deposition of A$\beta$ and hyperphosphorylation of Tau can also cause oxidative stress and exert its neurotoxic effects by producing free radicals in the brain in AD patients. Supplementation of exogenous antioxidants (such as vitamins, alkaloids, etc.) or improvement of endogenous antioxidant capacity plays an important role in delaying the pathological progression of AD.[14] In this study, the effect of donepezil hydrochloride on oxidative stress in AD rats was investigated by detecting oxidative stress indicators such as MDA, GPx, and SOD. SOD is an enzyme that scavenges superoxide anions in the body, and when oxidative damage occurs, the activity of SOD is inhibited, and the generated hydrogen peroxide cannot be removed in time, and reacts with other transition metals to form more toxic hydroxyl groups, which aggravates oxidative stress damage.[15] At the same time, SOD deficiency increases the level of A$\beta$, which promotes the occurrence of dementia in transgenic mice.[16] Therefore, SOD activity can be used as an important evaluation index for the degree of oxidative stress damage. Glutathione (GSH) plays a vital role in the maintenance of neuronal antioxidant defense system and redox homeostasis, and GSH is beneficial in maintaining the integrity of synaptosome structure and function, improving synaptic injury, and preventing nerve cell loss.[17] Glutathione peroxidase (GSH-Px) can promote the decomposition of glutathione, which can protect the body from oxidative free radical damage, and is a key enzyme in scavenging free radical antioxidants. Therefore, the activity of GSH-Px can reflect the ability of scavenging free radicals and serve as a biological index for detecting oxidative stress. The accumulation of MDA indicates that the activity of antioxidant enzymes in vivo leads to oxidative damage to lipids and DNA, which is a biomarker of oxidative stress, which can reflect the intensity and rate of lipid peroxidation in the body, and can indirectly reflect the degree of oxidative damage. The results of this study showed that, in comparison with the saline group, the levels of GPx and SOD in serum and hippocampal tissues in the DT group were significantly higher than those in the NS group, and the content of MDA was significantly lower than that in the NS group, suggesting that donepezil hydrochloride can improve AD by reducing oxidative stress damage.

Brain histopathological examination is the primary evaluation method for the degree of AD injury and treatment effect, which can directly reflect the morphological and functional status of nerve cells.[18] The hippocampus is an important area of the brain related to learning and memory function, and the decrease in the number of neuronal cells in the hippocampus will affect the formation of dendritic spines, which in turn will cause changes in synaptic plasticity, thereby affecting learning and memory abilities.[19] In this study, the pathological structure of hippocampal tissues was observed by means of HE staining, and the results showed that the number of neurons in the NC group of rats was abundant, neatly arranged, and distinct, with large and round nuclei and obvious nucleoli, and the number of neurons in the NS group was significantly reduced, and the neurons were arranged in an irregular manner, and there were phenomena such as nuclear consolidation and atrophy, indicating that the AD model was successfully prepared, and the number of neurons was increased after drug intervention in comparison with the NS group, indicating that donepezil hydrochloride had neuroprotective effects.

## 6. Conclusions

Donepezil hydrochloride can significantly improve the cognitive function, learning and memory abilities of AD rats, reduce neuroinflammation and oxidative stress response, and improve the pathological damage of brain tissues, which provides a basis for the clinical treatment of AD.

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Not applicable.

## Authors contributions

Wei-ling Song contributed to the study conception and design, data acquisition, analysis, and interpretation, manuscript drafting, review and editing; Jun Xue contributed to the study conception and design, data analysis, manuscript revision and review, academic leadership and guidance, financial support obtaining, manuscript editing; Xue-hui Wu and Jia-li He contributed to the data acquisition, manuscript revision and review.

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## Conflicts of Interest Disclosure

The authors have no conflicts of interest related to this article.

## Informed consent

Obtained.

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## PROVENANCE AND PEER REVIEW

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

## DATA SHARING STATEMENT

No additional data are available.

## OPEN ACCESS



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