Effect of Cuzhi liquid on learning and memory dysfunction in a mouse model of Alzheimer's disease

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**Abstract**

**OBJECTIVE:** To examine the effects of Cuzhi liquid on learning and memory abilities in a mouse model of Alzheimer’s disease (AD).

**METHODS:** One hundred mice were divided into the normal, AD model, piracetam group, Cuzhi liquid low dose and Cuzhi liquid high dose, each group 20 mice. The AD mouse model was induced by daily intraperitoneal injection of D-galactose and sodium nitrite. AD mice then received intragastric administration of piracetam or Cuzhi liquid for 60 d, and changes in learning and memory abilities were assessed using the water maze test. The activity of acetylcholinesterase (AchE) and monoamine oxidase (MAO), and the levels of nitrogen monoxidum (NO) and malonaldehyde (MDA), were measured in brain tissues. Amyloid protein deposition was assessed by methyl violet staining, and B-cell lymphoma-2 (Bcl-2) expression in the hippocampal cornus ammonis 1 region was detected by immunohistochemistry.

**RESULTS:** In the water maze test, the escape latency of the model group was longer than that of the normal group ($P < 0.01$). The escape latency of the three using drug treatment groups was significantly less than that of the normal group ($P < 0.05$). The activity of AchE and MAO, and the levels of NO and MDA, in the brain of the model group were significantly higher than that of the normal group ($P < 0.01$), but significantly reduced in the three drug treatment groups compared with the model group ($P < 0.05$). AchE activity showed a greater reduction in the two Cuzhi liquid groups compared with the piracetam group ($P < 0.01$), to levels similar to the normal group. There were no differences in MAO activity or NO levels between the three drug treatment groups, while MDA levels were reduced more in the high-dose Cuzhi liquid group compared with the other treatment groups ($P < 0.01$). Hippocampal Bcl-2 expression was significantly reduced in the model group compared with the normal group ($P < 0.01$), but significantly improved in the three drug treatment groups ($P < 0.05$). The high-dose Cuzhi liquid group showed a significantly greater recovery in Bcl-2 expression compared with the other treatment groups.

**CONCLUSION:** Cuzhi liquid can improve learning and memory impairment in an AD mouse model. The mechanism of action may relate to reduced AchE and MAO activity, and reduced NO and MDA levels, in the brain, and improved Bcl-2 expression, an inhibitor of apoptosis.
INTRODUCTION

Alzheimer’s disease (AD) is a progressive degenerative disease of unknown etiology, with predominant effects on intelligence, memory, sensory directional force, judgment, language and thinking ability, and personality changes. Approximately 37 million people worldwide have dementia, the majority of which is caused by AD, with an incidence of approximately 5% of men and 6% of women older than 60 years of age. Further, with the predicted increase in the aging population, the incidence of AD is expected to increase sharply over the next 20 years. In the present study, we examined the neuroprotective actions and potential mechanisms of action of Cuzhi liquid in a chemical AD animal model.

MATERIALS AND METHODS

Animals

A total of 100 healthy Kunming mice (50 male, 50 female) of 4-6 weeks of age and weighing 18-22 g were used in this study (Experimental Animal Center of Henan Province, Zhengzhou, China). Animals were housed in a standard environment at the Henan College of Traditional Chinese Medicine Laboratory Animal Center, with free access to food and water. After 1 week acclimatization, animals were randomly divided into the normal, AD model, piracetam group, Cuzhi liquid low dose (Pharmacy Department, People’s Hospital of Henan Province, Zhengzhou, China), and Cuzhi liquid high dose groups (Pharmacy Department, People’s Hospital of Henan Province, Zhengzhou, China) (n = 20 per group).

The AD animal model was established as previously reported. In brief, animals received intraperitoneal injection of 10 g/L D-galactose (120 mg/kg) and 10 g/L sodium nitrite (90 mg/kg) once daily for 60 d. Animals in the normal and AD model groups then received normal saline lavage (0.02 mL/g) for 60 d. Animals piracetam group, low dose Cuzhi fluid, and high dose Cuzhi fluid groups received 0.02 mL/g lavage mixed suspension (21 g/L) piracetam group, 0.02 mL/g Cuzhi fluid lavage, or 0.04 mL/g Cuzhi fluid lavage, respectively. All treatments were administered in the afternoon.

Ten mice (5 weeks old) were randomly selected from each group for water maze experiments (SMG type 2 water maze) (Experimental Center of Zhengzhou university, Zhengzhou, China). The water maze device was a 73 cm x 42 cm x 20 cm black organic glass tank, with a starting point and end point, four blind points, a depth of 10 cm, and a water temperature of (22 ± 1 °C). Water maze experiments were performed once daily. Mice were allowed to swim twice one day, and the second swim was used to record the time required to find the platform incubation period (escape; a maximum of 120 s was allowed to find the platform). Making them free practice, familiar with the environment during drug use end day, one blind side was opened in the first day and the time taken to reach the platform and the entry points to the platform were recorded. The number of open of blind sides was increased daily, until the fourth day, when the baffle was removed to provide four open blind sides, 5 d as 4 d. The escape latency period was measured every day. The 4 d study time for mice, and the fifth day memory for its time.

At the end of the behavioral experiments, the animals were rapidly euthanized by decapitation, and the brains were removed on an ice tray, the cerebellum and olfactory bulb removed, and the brains rinsed with saline (4 °C) and dried with filter paper. The brains were weighed, and then homogenized with cold physiological saline (volume: weight ratio 1: 9) in order to make a 10% brain tissue homogenate in an ice bath. The brain homogenates were centrifuged for 10 min at 3000 revolutions per min, and the supernatant removed and held at 4 °C until biochemical analysis of acetylcholinesterase (AchE) and MAO activity, and NO and MDA levels, using commercial kits (Jiangsu Feiya biology company, Naniing, China).

From the remaining mice, six animals per group were sedated with 40 mg/kg intraperitoneal injection of sodium pentobarbital anesthesia, and the brains perfusion fixed by rapidly opening the chest, exposing the heart, inserting an infusion needle into the left ventricle, cutting the right auricle, and then rapidly infusing 50-100 mL saline, followed by approximately 100 mL of 4% paraformaldehyde. The brains without cerebellum and the olfactory bulb were taken then immersion fixed in 4% paraformaldehyde solution for 24 h. The brains were then dehydrated and paraffin embedded, sectioned into continuous coronal slices (5 mm thick), and the sections containing the dentate gyrus of the hippocampus were stained with hematoxylin and eosin, methyl violet dye, or B-cell lymphoma-2 (Bcl-2) immunohistochemistry (Jiangsu Feiya biology company, Naniing, China).

Each section is randomly observed in five different high magnification visions (∗×400). According to the number of the Bcl-2 protein staining positive cells in each high magnification vision, mean five horizons of positive cells were calculated to represent the number of Bcl-2 protein positive cells number in a slice.

Statistical analysis

All data were analyzed with statistical software (SPSS v13.0; SPSS Inc., Statistics for Windows, Chicago, IL, USA). Data are the mean ± standard deviation ( ± )
RESULTS

Spatial learning test
The escape latency of the AD model mice was increased compared with the normal group on the first day of testing, while there were no differences between the groups on the second day. On the third day of testing, the escape latency of the AD model mice was significantly longer than that of the normal group (P < 0.01). Further, on the third and fourth days, the escape latency of the three treatment groups was significantly reduced compared with the model group (P < 0.05), although there were no differences between the three treatment groups. On the fifth day, the escape latency of the model group was significantly longer than that of the normal group, but was significant reduced in the three treatment groups compared with the model group (P < 0.01), to levels similar to normal animals (Table 1).

Biochemical analysis of brain tissue
AchE and MAO activity, and NO and MDA levels, in brain tissue homogenates were significantly increased in the AD model group compared with the normal group (P < 0.01, Table 2), but significantly reduced in the three treatment groups compared with the model group (P < 0.05). The two Cuzhi liquid groups showed a significantly greater reduction in AchE activity compared with the piracetam group (P < 0.01). There were no significant differences between two Cuzhi liquid groups and the normal group (P > 0.05). There were no differences in MAO activity and NO content between the three treatment groups. Finally, the high dose Cuzhi liquid group showed the greatest reduction in MDA content compared with the other two treatment groups (both P < 0.01).

Brain tissue morphology in the AD mouse model
Normal animals showed an ordered arrangement of neurons in the hippocampal cornus ammonis 1 (CA1) region at low magnification (Figure 1), with complete cellular structure at high magnification (Figure 2). By contrast, neurons in the AD model group were disordered and unclear, with scattered or continuous cellular degeneration and necrosis, cytoplasm deep staining, nuclear pyknosis, and degeneration of the nuclear membrane boundary. However, the structure of CA1 neurons was relatively preserved in the three drug groups compared with the model group.

Methyl violet staining
No β-amyloid protein (Aβ) deposition was observed in the hippocampus in the normal group (Figure 3). By

| Table 1 Escape latency in the water maze test in the various groups (x ± s) |
|-------------------|-----|-----|-----|-----|
| Group             | n   | 1 d | 2 d | 3 d |
| Normal            | 10  | 40±21 | 47±23 | 52±19 | 42±15 | 34±14 |
| Model             | 10  | 68±26 | 75±25 | 85±18 | 92±21 | 72±16 |
| Piracetam         | 10  | 50±29 | 54±19 | 60±20 | 61±23 | 38±15 |
| Low-dose Cuzhi liquid | 10  | 53±26 | 56±22 | 65±19 | 53±21 | 43±10 |
| High-dose Cuzhi liquid | 10  | 46±23 | 54±25 | 60±21 | 53±21 | 40±16 |

Notes: normal and AD model groups received normal saline lavage (0.02 mL/g) for 60 d. Piracetam group, low dose Cuzhi fluid, and high dose Cuzhi fluid groups received 0.02 mL/g lavage mixed suspension (21 g/L), 0.02 mL/g Cuzhi fluid lavage, and 0.04 mL/g Cuzhi fluid lavage, respectively. Comparisons between the three treatment groups (piracetam group, low dose Cuzhi fluid, and high dose Cuzhi fluid groups) and normal groups at different times (5 d as a course): Day 1: F = 1.593, P = 0.193; Day 2: F = 2.179, P = 0.087; Day 3: F = 4.050, P = 0.007; Day 4: F = 10.344, P = 0.000; Day 5: F = 11.059, P = 0.000. *P < 0.01, three treatment groups compared with the normal group; **P < 0.05, three treatment groups compared with the model group.

| Table 2 Effect of Cuzhi liquid on acetylcholinesterase (AchE) activity, monamine oxidase (MAO) activity, nitrogen monoxide (NO) levels, and malonaldehyde (MDA) content in brain tissue homogenates (x ± s) |
|-------------------|-----|-----|-----|-----|
| Group             | n   | AchE (U/mgprot) | MAO (U/mgprot) | NO (μmol/gprot) | MDA (nmol/gprot) |
| Normal            | 10  | 0.93±0.21 | 4.24±0.65 | 4.20±0.61 | 21.11±2.78 |
| Model             | 10  | 1.60±0.27 | 6.72±0.88 | 5.48±0.87 | 38.69±6.42 |
| Piracetam         | 10  | 1.36±0.19 | 4.59±0.74 | 4.29±0.89 | 31.83±6.37 |
| Low dose Cuzhi liquid | 10  | 0.99±0.22 | 4.66±0.88 | 4.25±0.89 | 31.59±5.06 |
| High dose Cuzhi liquid | 10  | 1.05±0.22 | 4.55±0.75 | 4.23±0.82 | 26.19±6.39 |

Notes: normal and AD model groups received normal saline lavage (0.02 mL/g) for 60 d. Piracetam group, low dose Cuzhi fluid, and high dose Cuzhi fluid groups received 0.02 mL/g lavage mixed suspension (21 g/L), 0.02 mL/g Cuzhi fluid lavage, and 0.04 mL/g Cuzhi fluid lavage, respectively. Comparison of biochemical indexes between the different groups: AchE: F = 15.972, P = 0.000; MAO: F = 16.173, P = 0.000; NO: F = 4.575, P = 0.003; MDA: F = 13.997, P = 0.000. *P < 0.01, compared with the normal group; **P < 0.01, compared with the model group; ***P < 0.05, compared with the model group; ****P < 0.01, compared with the piracetam group; *****P < 0.05, compared with the normal group; ******P < 0.05, compared with the piracetam group.
duce learning and memory abilities in mice, which was a combination of sodium nitrite and galactose can re- 

In the present study, we found that administration of a 

DISCUSSION 

The number of Bcl-2-positive cells in the CA1 region of the hippocampus was significantly reduced in the AD model group compared with the normal group (Table 3, Figure 4), but significantly increased in the three treatment groups (all \( P < 0.05 \)), indicating reduced 

Expression of Bcl-2 positive cells in the hippocampal CA1 region 

The number of Bcl-2-positive cells in the CA1 region of the hippocampus was significantly reduced in the AD model group compared with the normal group (Table 3, Figure 4), but significantly increased in the three treatment groups (all \( P < 0.05 \)), indicating reduced neuronal apoptosis. The high dose Cuzhi liquid group showed the greatest protective effects, with significantly higher numbers of Bcl-2-positive cells compared with the piracetam group \( (P < 0.05) \). 

Associated with disordered arrangement, degenerative changes, and apoptosis of hippocampal neurons, amyloid deposition, and elevated AchE activity, \(^{22} \) MAO activity, NO levels, and MDA levels in the brain. Thus, this model reproduces some of the functional and pathological aspects of AD. We also found that treatment with Cuzhi liquid can reduce AchE activity, MAO activity, NO levels, and MDA levels in the brain, increase the cellular expression of the anti-apoptotic 

Table 3 Effect of Cuzhi liquid on numbers of Bcl-2-positive cells in the hippocampal cornus ammonis 1 area in Alzheimer’s disease (AD) mice \( (n = 5) \) 

<table>
<thead>
<tr>
<th>Group</th>
<th>Bcl-2-positive cells (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>28 ± 7</td>
</tr>
<tr>
<td>Model</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>Piracetam</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>Low dose Cuzhi liquid</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>High dose Cuzhi liquid</td>
<td>23 ± 7</td>
</tr>
</tbody>
</table>

Notes: normal and AD model groups received normal saline lavage (0.02 mL/g) for 60 d. Piracetam group, low dose Cuzhi fluid, and high dose Cuzhi fluid groups received 0.02 mL/g lavage mixed suspension (21 g/L), 0.02 mL/g Cuzhi fluid lavage, and 0.04 mL/g Cuzhi fluid lavage, respectively. Comparison between the groups: \( F = 10.772, P = 0.0001 \). \( ^{2} P < 0.01 \), compared with the normal group: \( ^{3} P < 0.05 \), compared with the model group: \( ^{4} P < 0.01 \), compared with the model group: \( ^{5} P < 0.05 \), compared with the piracetam group. Bcl-2: B-cell lymphoma-2.
Bcl-2 protein and neuronal survival in the hippocampal CA1 region, and improve learning and memory dysfunction in this AD mouse model. According to the pathogenesis of AD and previous reports on AD treatment, we prepared a Cuzhi liquid using walnuts, the rhizome of Dihuang (Radix Rehmanniae), Shanyao (Rhizoma Dioscoreae Opposita), Shan-zhuuyu (Fructus Corni), Dangshen (Radix Codonopsis), Wuweizi (Fructus Schisandrae Chinensis), malayeta scurfpea fruit, Yuanzhi (Radix Pseudocydoniae), Hehuanshi (Cortex Althaeae), 10 liquid licorice drugs, and walnut meat kidney marrow. Treatment mechanisms of Cuzhi liquid is as follows: the rhizome of Dihuang (Radix Rehmanniae) nourishing Yin and filling pulpy; Shan-zhuuyu (Fructus Corni) benefit spleen and kidney; Shanyao (Rhizoma Dioscoreae Opposita) tonifying Qi and nourishing Yin, lung, kidney, and spleen; Dangshen (Radix Codonopsis) tonifying spleen and replenishing Qi and nourishing blood; malayeta scurfpea fruit Yang fixing sperm for princes; Yuanzhi (Radix Pseudocydoniae) calming nerves, expectorant goose; Hehuanshi (Cortex Althaeae) nerves resolve depression; Wuweizi (Fructus Schisandrae Chinensis) calming nerves, licorice to reconcile the medicine. Reasonable compatibility, altogether plays brain puzzle, filling lean pulp, tonifying spleen and kidney, nourishing heart, and resolving depression nerves.

In conclusion, our findings suggest that Cuzhi liquid can improve learning and memory impairment in an AD-like mouse model. Further, the mechanism of action may relate to reducing AchE activity, MAO activity, NO levels, and MDA levels in the brain, and improving the expression of Bcl-2, an anti-apoptotic protein.

REFERENCES

Figure 3 β-amyloid protein (Aβ) deposition in the hippocampus among different groups (methyl violet staining, ×200) A: the normal group; there is no Aβ deposition in the hippocampus area; B, C: model groups: there are a lot of Aβ deposition; D: piracetam group: there is a little of Aβ deposition; E: low dose group: there is a little of Aβ deposition; F: high dose group: there is a little of Aβ deposition. Piracetam group and high dose group are decreased obviously. Normal and AD model groups received normal saline lavage (0.02 mL/g) for 60 d. Piracetam group, low dose Cuzhi fluid, and high dose Cuzhi fluid groups received 0.02 mL/g lavage mixed suspension (21 g/L), 0.02 mL/g Cuzhi fluid lavage, and 0.04 mL/g Cuzhi fluid lavage, respectively.

Figure 4 Expressions of Bcl-2 protein in the hippocampal cornus ammonis 1 region among different groups (immunohistochemical staining, × 400) A: normal group, the number of expressions of Bcl-2 protein is normal; B: model group, the number of expressions of Bcl-2 protein is significantly reduced; C: piracetam group; D: walnut extract low dose group; E: walnut extract high dose group. C, D, E are three treatment groups: the number of expressions of Bcl-2 protein is significantly increased, especially high dose group. Normal and AD model groups received normal saline lavage (0.02 mL/g) for 60 d. Piracetam group, low dose Cuzhi fluid, and high dose Cuzhi fluid groups received 0.02 mL/g lavage mixed suspension (21 g/L), 0.02 mL/g Cuzhi fluid lavage, and 0.04 mL/g Cuzhi fluid lavage, respectively. Bcl-2: B-cell lymphoma-2.


