Effects of Chinese herbal medicine Yinsiwei compound on spatial learning and memory ability and the ultrastructure of hippocampal neurons in a rat model of sporadic Alzheimer disease

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Objective: To study the effects of Chinese herbal medicine Yinsiwei compound (YSW) on spatial learning and memory ability in rats with sporadic Alzheimer disease (SAD) and the ultrastructural basis of the hippocampal neurons.

Methods: A rat model of SAD was established by intracerebroventricular injection of streptozotocin. The rats were divided into six groups: sham-operation group, model group, donepezil control group, and YSW low, medium and high dose groups. Drug interventions were started on the 21st day after modeling and each treatment group was given the corresponding drugs by gavage for two months. Meanwhile, the model group and the sham-operation group were given the same volume of distilled water by gavage once a day for two months. The Morris water maze was adopted to test spatial learning and memory ability of the rats. The place navigation test and the spatial probe test were conducted. The escape latency, total swimming distance and swimming time in the target quadrant of the rats were recorded. Also, the hippocampus tissues of rats were taken out and the ultrastructure of hippocampus neurons were observed by an electron microscope.

Results: In the place navigation test, compared with the model group, the mean escape latency and the total swimming distance of the donepezil group and the YSW low, medium and high dose groups were significantly shortened ($P<0.05$ or $P<0.01$). In the space probe test, the swimming time of each treatment group in the target quadrant was significantly longer than that of the model group ($P<0.05$ or $P<0.01$). For most of the test period, the donepezil group had no
significant change compared with the YSW low-, medium and high dose groups, respectively. The ultrastructure of the hippocampus neurons under the electron microscope also confirmed the efficacy of the drug treatment.

_**Conclusion:**_ Chinese herbal medicine YSW compound can improve spatial learning and memory impairment of rats with SAD. The ultrastructural basis may be that it can protect the microtubule structures of hippocampal neurons and prevent nerve axons from being damaged.

**Keywords:** Alzheimer disease; drugs; Chinese herbal; maze learning; learning disorders; memory disorders; hippocampus; animal experimentation; rats

Alzheimer disease (AD) can be divided into two types, familial and sporadic. AD accounts for approximately 65% of all the dementia cases\(^1\), which has become the fourth killer of the elderly following cardiovascular diseases, malignancy and stroke. Among them, sporadic Alzheimer disease (SAD) accounts for 95% of AD. Familial AD is often caused by gene mutation, but SAD is mainly due to genetic risk factors, aging as well as environmental risk factors\(^2\), which is the most controllable type of AD by drug intervention.

Yinsiwei compound (YSW) is the effective extracts by recipe construction and dose optimization based on the empirical prescription from the famous doctor Chen Shi-duo of the Qing Dynasty for treating AD. Our research systematically studied the function of YSW to treat cognitive impairment of SAD and its possible mechanism. This article reported part of the research, focusing on observation and discussion of the effects of YSW on learning and memory in SAD model rats and on the ultrastructure of the hippocampal neurons.

1 Materials and methods

1.1 Experimental animals and grouping A total of 84 healthy male Sprague-Dawley rats (specific pathogen free), weighing (270±20) g, were provided by the Experimental Animal Center of Fourth Military Medical University (certification number of the experimental animals: 0034642). The rats were in the state of free intake of food and water during the experiment (except for pre-operative fasting), with room temperature of 22 to 26 °C and humidity of 30% to 40%. The rats were randomly divided into sham-operation group (S), model group (M), donepezil group (D), YSW low dose group (YL), YSW medium dose group (YM) and YSW high dose group (YH) with 14 rats in each group.

1.2 SAD modeling Based on the reported methods\(^3\), all the rats were fixed in the stereotaxic apparatus after anesthetized by 10% chloral hydrate (3.8 mL/kg body weight). Their skins over the skull were routinely disinfected. The median sagittal plane of the skull was cut and the periostem was separated from the skull; the skull was then drilled and the dura was exposed. Except the S group, rats in the other five groups were injected with 18 μL streptozotocin (STZ) each (STZ was dissolved in artificial cerebrospinal fluid before injection and its concentration was 25 mg/mL) on each side of lateral ventricle by using a Hamilton syringe. Based on The Rat Brain in Stereotaxic Coordinates by Paxinos et al.\(^4\), the coordinates were 1.5 mm behind the anterior fontanel, 1.5 mm lateral to the sagittal suture and 3.5 mm beneath the scurf. Reduplicate injection was conducted on the 3rd day and the dose was the same as before. The S group rats were injected with the same amount of artificial cerebrospinal fluid. After modeling, the rats were routinely fed for 21 d, and then drug interventions were conducted. During the process of modeling and feeding, mortality of rats was about 19.2%, which was roughly balanced in each group during the preliminary experiment as well as the formal experiment.

1.3 Drug administration YSW is composed of Renshen (Radix Ginseng), Banxia (Rhizoma Pinelliae), Fushen (Poria Cum Radix Pini), Fuzi (Radix Aconiti Lateralis Preparata), Shichangpu (Rhizoma Acori Tatarinovii) and some other Chinese herbs. The particles of the Chinese herb extracts were provided by the China Resources Sanjui Medical & Pharmaceutical Co., Ltd. (batch number: 0908032), of which 29 g is equivalent to the original prescription of crude drug 169 g. During the experiment, the particles were dissolved and diluted by 60 °C double-distilled water and made into different concentrations of low, medium and high doses (calculated by crude drug dose for rats: YSW low dose 7.61 g/(kg · d); YSW medium dose 15.21 g/(kg · d); YSW high dose 30.42 g/(kg · d)). The low-, medium- and high-dose YSW was given to rats in the YL, YM and YH groups at 7.5 mL/(kg · d) by gavage, respectively. Donepezil, produced by Xi’an Haixin Pharmaceutical Co., Ltd. (batch number: 091201), was bought from the pharmacy of Xijing Hospital affiliated to Fourth Military Medical University and its dose was 0.22 mg/(kg · d). Donepezil was given to rats in the D group at 7.5 mL/(kg · d) by gavage. Rats in M group and S group were administered with the same volume of double distilled water by gavage. All the rats were administered once a day for two months.

1.4 Ethology test The Morris water maze was used in this experiment\(^5\). The place navigation test was used to measure the spatial learning and memory ability of the animals in the water maze. The animals were allowed to swim freely for 2 min before the test. During the test, the animals were trained twice daily each for 120 s for 5 d. A quadrant adjacent to the quadrant in which the platform is located was selected to put the rats facing the wall
into the water and the time for them to find the target platform and climb up was recorded, respectively. This time span was called escape latency. Swimming distance was also recorded for each rat. If the rat did not find the platform in 120 s, it will be brought out of the water to the platform by the experimenter and stays there for 20 s. For such rats the escape latency was recorded as 120 s. Spatial probe test was used to measure the memory ability of rats for memorizing the accurate location of the platform, namely, memory retention. The platform was removed on the 6th day and the rat was put into the water from any place to swim for 120 s and the swimming time of rat in the target quadrant (the quadrant in which the platform is located) and other quadrants was recorded, respectively.

1.5 Electron microscope observation At the end of ethology test, rats were sacrificed by cutting off the neck under anesthesia. The fresh hippocampus tissues were taken out and each one was cut into 1 mm² tissue blocks on ice cubes. The tissue blocks were put into 2.5% glutaraldehyde fixative for 2 h until they sink down, then they were washed by 0.01 mmol/L phosphate buffered solution (PBS) for three times and kept in 0.01 mmol/L PBS at room temperature. Electron microscope observation was conducted by using a transmission electron microscope (JEM-2000EX, JEOL Ltd., Japan).

1.6 Statistical analysis The gained data were expressed as X±s. SPSS 17.0 statistics software was used for statistical analysis. Comparisons among groups were conducted by one-way analysis of variance (ANOVA), followed by least-significant difference (LSD) t test. P<0.05 was considered to be statistically significant.

2 Results
2.1 Results of ethology test
2.1.1 Place navigation experiment During the five-day place navigation test, the average escape latency and total swimming distance of rats in each group gradually decreased, which indicated that the ability of the rats in seeking the platform was improved in the previous learning and training process. On the 1st day of training, the average escape latency and total swimming distance of the rats in the M group and the S group had no significant difference, but they had a remarkable difference in the following 4 d (P<0.05 or P<0.01). From the 3rd day, the average escape latency and total swimming distance of the D group and the YL, YM and YH groups showed a significant difference compared with the M group (P<0.05 or P<0.01). On the 2nd day, it showed that the average escape latency and total swimming distance of the YH group had a significant difference compared with the D group (P<0.05). The D group had no significant difference in comparison with the YL, YM and YH groups in other time points, respectively (Table 1, Figure 1, Table 2, Figure 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Average escape latency (s)</th>
</tr>
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<tbody>
<tr>
<td>S</td>
<td>11</td>
<td>75.74±25.03 31.37±10.75 20.45±10.14 12.91±3.10 10.67±3.59</td>
</tr>
<tr>
<td>M</td>
<td>12</td>
<td>84.08±7.92 88.08±25.02* 50.36±31.85* 47.62±30.50* 40.23±34.12*</td>
</tr>
<tr>
<td>YL</td>
<td>11</td>
<td>95.09±45.99 50.39±28.84 25.89±20.45 23.52±11.15 15.75±13.30</td>
</tr>
<tr>
<td>YM</td>
<td>11</td>
<td>55.54±19.81 40.59±14.16 26.52±21.43 30.58±27.75 19.95±23.07</td>
</tr>
<tr>
<td>YH</td>
<td>11</td>
<td>55.32±20.85 27.07±10.17 19.84±11.89 16.84±12.38 11.12±7.38</td>
</tr>
</tbody>
</table>

* P<0.01 vs S group; # P<0.05, & P<0.01 vs M group; # P<0.05 vs D group. S: sham-operation; M: modell; D: donepezil; YL: Yiniseiwe compound of low dose; YM: Yiniseiwe compound of medium dose; YH: Yiniseiwe compound of high dose.

Figure 1 Comparison of average escape latency in the place navigation test of rats in each group
YL: Yiniseiwe compound of low dose; YM: Yiniseiwe compound of medium dose; YH: Yiniseiwe compound of high dose.
2.1.2 Spatial probe test. During the spatial probe test, the rats in the M group stayed longer in the outer region of the target quadrant but had fewer activities around the target quadrant compared with the S group ($P < 0.05$). Compared with the M group, the swimming time in the target quadrant of rats in each treatment group became significantly longer ($P < 0.05$ or $P < 0.01$). The D group had no significant difference in comparison with the YL, YM and YH groups, respectively (Table 3).

2.2 Ultrastructural changes in the hippocampal neurons of rats. The hippocampal neurons of rats in the S group formed regularly with smooth nuclear membrane, clear nucleolus and chromatin distribution. The microtubules of neuronal axons were arranged neatly in fascicular pattern with no fracture or dissolution (Figure 3A). The hippocampal neurons of rats in the M group showed ischemic changes, with heterochromatin increased. The structure of organelles was damaged in varying degree. The microtubules of neuronal axons had obvious fracture or dissolution, disappeared in most regions and arranged in disorder (Figure 3B). The pathological cells had significant improvement in all the treatment groups. The microtubules of hippocampal neurons of rats had no obvious fracture or dissolution and arranged neatly with clear structure of axons, which were similar to the S group (Figure 3C to 3F).

### Table 3 Comparison of swimming time around the target quadrant of rats in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Swimming time around the target quadrant (±s, second)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>11</td>
<td>53.49±3.57</td>
</tr>
<tr>
<td>M</td>
<td>12</td>
<td>15.92±5.16**</td>
</tr>
<tr>
<td>D</td>
<td>12</td>
<td>41.02±10.75A</td>
</tr>
<tr>
<td>YL</td>
<td>11</td>
<td>39.01±13.97A</td>
</tr>
<tr>
<td>YM</td>
<td>11</td>
<td>52.81±4.30A</td>
</tr>
<tr>
<td>YH</td>
<td>11</td>
<td>55.45±5.32A</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, vs S group; * $P < 0.05$, ** $P < 0.01$, vs M group; YL: Yinsuwei compound of low dose; YM: Yinsuwei compound of medium dose; YH: Yinsuwei compound of high dose.
3 Discussion

In general, the pathological features of AD are mainly degenerative changes of the hippocampus and cerebral cortex neurons, intracellular neurofibrillary tangles (NFT) and extracellular senile plaque (SP) deposition. The number of NFT has a positive correlation with the degree of dementia, which is considered to be the pathological basis of neuronal degeneration in AD patients[6]. The main component of NFT is paired helical filaments, which are formed by abnormal hyperphosphorylation of protein tau aggregation. Recent studies showed that abnormal hyperphosphorylation of microtubule-associated tau protein not only makes itself lose the normal bioactivity (which is to catalyze microtubule assembly and stabilize structure of microtubules), but also changes itself into cytolytic molecules and deposits itself into NFT[10]. In addition, the abnormal hyperphosphorylation of tau protein can hunt for the normal microtubule-associated protein, resulting in breakdown of microtubules and degeneration of axons, and eventually leading to serious damage in cognitive function of AD patients. Therefore, in order to protect structure of the microtubules and prevent the axons from denaturation and injury, the key strategy to prevent and cure SAD is to inhibit abnormal hyperphosphorylation of tau protein and the formation of NFT.

AD falls into the category of dementia in traditional Chinese medicine (TCM). Abundant clinical experience has been accumulated in the prevention and treatment of this disease with further digging of the ancient literature in this field. The curative effects of some famous TCM prescriptions have been tested in long-term clinical practices. Therefore, it is quite necessary to do some detailed and further experimental researches to reveal the mechanisms and provide the example for prevention and treatment of complex diseases by TCM.

The YSW used in this study evolved out of the famous prescription of treating dementia proposed by eminent doctor Chen Shiduo of Qing Dynasty. The original prescription is composed of Renshen, Banxia, Fushen, Fuzi, Shichangpu and some other Chinese herbs. YSW is a compound of the extracts of effective components after optimization and refining the original prescription. Based on the TCM therapeutic principle described as tonifying qi and warming yang and expelling stagnation and removing phlegm, the whole prescription was worked out for addressing both symptoms and root causes. The nature of this compound is ascending yang qi, dispelling pathogenic yin, expelling stagnation, removing phlegm and balancing yin and yang.

The SAD model, established by intracerebroventricular injection of streptozotocin (ICV-STZ), is internationally recognized and adopted in this experiment. Blokland et al.[11] found that choline acetyltransferase activity of the hippocampal neurons of rats can be reduced by intraventricular injection of STZ, which gives rise to the decline of spatial learning and memory ability of rats. Since then, the basic research on establishing the SAD model by ICV-STZ appeals to many scholars. In 2005, de la Monte et al.[13] used the method of ICV-STZ to block the autophosphorylation of insulin receptor and the activity of intrinsic tyrosine
kinase, and to damage the signal transduction pathways of insulin and insulin-like growth factor. It was found that a minute amount of STZ did not cause rise of glucose or change of structure of pancreas or immunocompetence of insulin, but it can lead to reduction of the brain volume, loss of neurons, gliosis, more β-amyloid deposition, hyperphosphorylation of tau protein, ubiquitination and so on. It successfully established the model of simulating the pathological features of SAD, so it earned universal acceptance in academic circles. The animals with SAD, established by ICV-STZ, had obvious barriers in learning and memorizing on the 21st day after modeling. In recent years, this model has been widely used as SAD and “type 3 diabetes” model, and applied in the research of brain energy metabolism of AD and phosphorylation of tau protein. The Morris water maze system used in this experiment was designed by the British psychologist Morris in the early 1980s, which is used for the research of mechanisms of learning and memory that is directly related to the functions of the hippocampus. The system can exactly reflect the spatial learning and memory ability of animals, and it is an important tool for testing animal ethology.

The results of the present study showed that ICV-STZ in rats can cause disorders of spatial learning and memory and the M group had a significant difference from the S group \( (P < 0.01) \). In the place navigation test for testing the learning ability of rats, the YSW compound of low, medium and high doses could significantly shorten the swimming time and the total swimming distance of the SAD rats in the Morris water maze. In the spatial probe test for testing memory ability of rats, the YSW compound of low, medium and high doses could significantly prolong the swimming time of rats in the target quadrant, increase their activities around the platform and improve their strategy for searching the target. In most cases, the YL, YM and YH groups had no significant difference in comparison with the D group. The above results indicated that both the extracts of YSW compound and donepezil can improve the spatial learning and memory ability in SAD model rats. According to the ultrastructure of the hippocampal neurons, the hippocampal neuronal axons remained intact and arranged neatly in fascicular pattern in the S group. In the M group, the hippocampal neuronal axons had obvious fracture or dissolution, and the microtubules disappeared or were disordered in most regions. In the D group and the three YSW compound treatment groups, the hippocampal neuronal axons arranged regularly without obvious fracture or dissolution. The above results showed that ICV-STZ can simulate the spatial learning and memory impairment resembling SAD and YSW compound can significantly improve the learning and memory ability of SAD rats. Its micro-morphological basis was related to protecting the microtubule structure and preventing the axons from being damaged.

YSW is the compound of extracts of effective components of the herbs, including high amounts of ginseng saponin. Ginseng saponin can boost the acetylcholine level in the brain and the number of M-cholinergic receptor, increase the efficacy and plasticity of synapse, inhibit cell apoptosis and necrosis, and promote neurogenesis in the hippocampus. The herbs such as Banxia, Fushen, Fuzi and Shichangpu in this prescription, which are frequently used in treating AD in TCM, are in the nature of dispersing and relieving effects. It can be speculated that the effect of the prescription in improving learning and memory ability may be related to the above herbs and their effective ingredients. The deep mechanism of its effect in improving learning and memory impairment of SAD is worth studying further.

4 Acknowledgements

The authors want to thank Professor Jin-zhou Tian for funding this project. We would also thank the staff at the Animal Care Facility of Shaanxi University of Chinese Medicine and Fu-ling Xin, Min Li, Na Zhao for the assistance in the animal experiment. We would like to thank the staff at the Electron Microscopy Center of Fourth Military Medical University for the assistance in electron microscope observation.

REFERENCES


复方银思维对散发性老年痴呆模型大鼠学习记忆能力的影响
及其海马神经元超微形态学基础

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目的: 探讨中药复方银思维对散发性老年痴呆（sporadic Alzheimer disease, SAD）模型大鼠空间学习记忆功能障碍的作用及其海马神经元超微形体系学基础。

方法: 采用侧脑室注射链脲佐菌素方法制备 SAD 模型。实验大鼠分为假手术组、模型组、多奈哌齐组和银思维低、中、高剂量组共 6 组。于造模第 21 天开始治疗，各治疗组给予相应的药物灌胃，模型组及假手术组给予等体积的蒸馏水灌胃，各组均每日给药 1 次，共 2 个月。治疗结束后，以 Morris 水迷宫定位航行实验及空间探索实验进行大鼠空间学习记忆能力测试，测试完毕处死大鼠并取出海马组织，电子显微镜观察海马神经元超微结构。

结果: 在定位航行实验中，多奈哌齐组以及银思维低、中、高剂量组与模型组相比，平均逃避潜伏期及总游泳距离均显著缩短（P<0.05 或 P<0.01）。在空间探索实验中，与模型组相比，各治疗组在目标象限活动时间明显延长（P<0.05 或 P<0.01）。在多数测试时段中，多奈哌齐组与银思维低、中、高剂量组组间比较差异均无统计学意义。脑组织海马神经元超微结构观察也证实了药物干预的作用。

结论: 中药复方银思维能明显改善 SAD 模型大鼠的空间学习记忆功能障碍。其作用的超微形态学基础可能与保护海马神经元超微结构，防止轴突损伤有关。

关键词: 阿尔茨海默病; 中草药; 迷宫学习; 学习障碍; 记忆障碍; 海马; 动物实验; 大鼠