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Original article

Ameliorating effect of *Alpinia oxyphylla—Schisandra chinensis* herb pair on cognitive impairment in a mouse model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia. In our previous study, we found both *Alpinia oxyphylla* and *Schisandra chinensis* can improve the cognitive function of AD. To investigate whether the *Alpinia oxyphylla* – *Schisandra chinensis* herb pair (ASHP) has ameliorating effect on cognitive impairment, we used scopolamine to induce learning and memory impairments, as a mouse model of AD. Subsequently, we carried out Y-maze test and Morris water maze test to observe the behavior of mice. Finally, the level of Acetylcholine (Ach) and muscarinic receptor (M1) receptors, the activity of choline acetyltransferase (ChAT) and acetyl cholinesterase (AChE) were measured by commercial assay kits and ELISA kit. And we used hematoxylin-eosin (HE) staining to check the changes in cortex and the CA1 region of hippocampus. ASHP significantly protected against learning and memory impairments induced by scopolamine in Y-maze test and Morris water maze test. Besides, ASHP was able to increase the level of ACh and M1 receptors, and decrease the activity of AChE, but did not significantly affect the activity of ChAT. In addition, from the results of histopathological examination, we speculated ASHP may have neuroprotective effects. This study provided an experimental basis for further study of *Alpinia oxyphylla* − *Schisandra chinensis* herb pair in AD therapy.

1. Introduction

As the most common cause of dementia, Alzheimer's disease (AD) is characterized by the damage with memory, language, problem-solving and other cognitive skills that affect a person's ability to perform everyday activities [1]. Past findings had shown that accumulation of abnormally folded Aβ and Tau proteins in amyloid plaques and neuronal tangles are causally related to neurodegenerative processes in patients' brains [2]. Besides that, hypertension, diabetes, genetics and other factors are also considered as AD risk factors [3]. Of all these hypotheses, the cholinergic hypothesis was the first theory to be put forward, based on a series of experiments and many long-term clinical observations [4–8]. Furthermore, the animal models built up under this hypothesis were widely used [9], and the most commonly used drugs for AD are acetyl cholinesterase inhibitors (AChEIs).

Acetylcholine (ACh) is a neurotransmitter which is most closely related to human learning and memory. Currently, there is a general consensus that dysfunction of the cholinergic system is associated with a declined in ACh levels. Furthermore, there are several interactions between ACh and markers of AD pathology [10]. Such as, Aβ can damage the release of ACh [11], conversely, ACh has an impact on the production process of Aβ and reduces Tau protein phosphorylation [12].

The main position of Ach synthesis is cholinergic nerve ending; choline acetyltransferase (ChAT) catalyzes choline and acetyl coenzyme A, thereby synthesizing ACh. So, ChAT is a key constituent of cholinergic neurons required for ACh synthesis, and in AD, the activity of ChAT decreased [13,14]. It has been reported that ChAT decreases amyloid precursor protein (APP) metabolism in neurons of APP/PS1 transgenic mice [15]. In addition to ChAT, acetyl cholinesterase (AChE) is also a key enzyme associated with ACh levels. AChE is an enzyme that catalyzes the hydrolysis of ACh, thus associated with the reduction of

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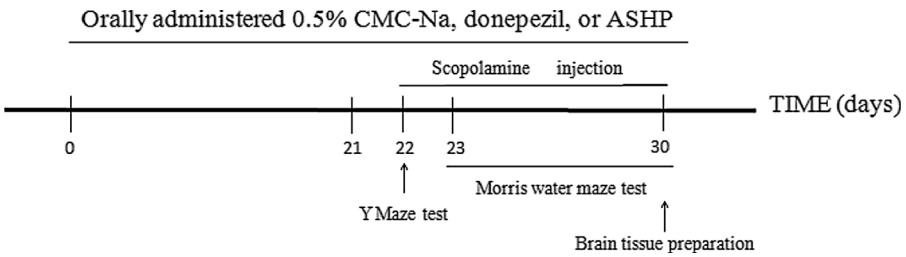

Fig. 1. The experimental schedule.

ACh level. Therefore, AChEIs has become one of the focuses of the anti-AD drugs research. Most of FDA approved anti-AD drugs, such as donepezil, galantamine, rivastigmine belong to AChEIs.

In brains, both nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) are bind to ACh, and these two receptors are related to cognitive function in AD. As members of the G Protein-coupled receptors (GPCRs), mAChRs are widely distributed in the brain and other organs, and are composed of a family of five receptor subtypes (M1, M2, M3, M4 and M5). Among them, M1 is an important subtype interacts with AD, for it is the predominant subtype found in the cerebral cortex and is involved in the control of cognitive functions [16].

Scopolamine, a muscarinic cholinergic receptor antagonist, has been reported that it can impair the learning and memory ability of rats [17]. In addition to that, microdosing of scopolamine can induce impairment in memory and information processing in healthy adults, which is similar to AD patients [18]. This model is frequently employed as an AD model to inspect whether the drugs tested can improve cognitive function [19–21].

In the Prescriptions for Universal Relief, there is a Chinese prescription called Yizhiwuweiwan, has been used as a treatment of liver and kidney deficiencies in China.. According to the traditional Chinese medicine theory, liver and kidney asthenia is one of the important causes of dementia. Both *Alpinia oxyphylla* and *Schisandra chinensis* are the main ingredients of this Chinese prescription. *Alpinia oxyphylla* as a traditional Chinese medicine has been used for kidney, spleen and stomach for thousands of years, its fruit called “Yizhi” in China. In our previous research, we found that *A. oxyphylla* ethanol extract, n-butanol extract and 5-Hydroxymethylfurfural (a main effective compound of *A. oxyphylla* ethanol extract) could ameliorate cognitive impairment in a mouse model of AD [22,23]. Similarly, as an important traditional medicinal material, *Schisandra chinensis*, which fruit called “Wuweizi” in China, has been extensively studied. Besides relieving diseases associated with the lungs, heart and kidneys, its effective compounds Schisandrin C and Schisantherin A have been reported are useful for cognitive decline [24,25].

So based on our previous experiment, in this experiment, we hypothesized that *Alpinia oxyphylla* – *Schisandra chinensis* herb pair (ASHP) could ameliorate the cognitive impairment in scopolamine induced animal model of AD and investigate its possible mechanism of action.

2. Materials and methods

2.1. Materials

A. oxyphylla and *S. chinensis* were purchased from Shenyang Tongrentang Drug Co., Ltd. (Shenyang, China) and identified by Professor Ying Jia (School of Functional Food and wine, Shenyang Pharmaceutical University) according to the guidelines of the Chinese Pharmacopoeia (2015). Then, the fruits of *A. oxyphylla* and *S. chinensis* (1:1) were exhaustively extracted with 95% ethanol at reflux for 2 h 3 times. After concentration in a vacuum, the residue was suspended in 0.5% sodium carboxymethylcellulose (CMC-Na) at a certain concentration of 120 mg/mL.

Scopolamine hydrobromide was obtained from Melone Pharmaceutical Co. (Dalian, China). Donepezil was obtained from Wanbang Pharmaceutical Company (Zhejiang, China). Commercial kits used for determination of ACh, ChAT and AChE were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). ELISA assay kits used for determination of M1 receptors is purchased from Shanghai enzyme bio Co., Ltd. (Shanghai, China).

2.2. Animals

Male 10-week-old KM mice, weighing 25–30 g were purchased from the Central Animal House of Shenyang Pharmaceutical University (Shenyang, China), were given food and water ad libitum and maintained in a colony room at 22 ± 2 °C with 45% ± 10% humidity under a 12:12 h light/dark cycle (lights on 07:00 to 19:00 h). Experiment was carried out in compliance with the National Institutes of Health and institutional guidelines for the humane care of animals and was approved by the Animal Care Committee of Shenyang Pharmaceutical University.

2.3. Experimental design

The mice were divided into 4 groups randomly and 10 in each group: control group, vehicle group (0.5% CMC-Na), donepezil group (3 mg/kg·d donepezil), ASHP group (1200 mg/kg d ASHP), except the control group, the rest of groups were orally administered for 30 days. From the 22nd day, 30 min after administration, except the control group injection of saline, the rest of groups peritoneal injection of scopolamine (3 mg/kg d) for 9 days. 30 min after injection, behavioral experiments were conducted. Brief experimental design is explained in Fig. 1.

2.4. Y-maze test

Y-maze test was carried out on the 22nd day to assess immediate spatial working memory which is a form of short-term memory [26]. The Y maze is comprised of three arms (90 cm long × 12 cm wide × 12 cm high) that are at 120° angles from each other, labeled A, B, C clockwise, and was placed in a room without noise. 24 h before testing, the mice were placed in the test room to adapt the environment. Animals were initially placed within one arm, and the sequence and number of arm entries were recorded manually for each one over an 8-min period. The alternation score (%) for each mouse was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100 as shown by the following equation: % Alternation = [(Number of alternations)/(Total arm entries − 2)] × 100.

2.5. Morris water maze test

Morris water maze was used to assess the recognition memory and spatial learning ability of the mice [27]. A circular pool (90 cm diameter and 30 cm height) filled with water (22 °C ± 1) to a depth of 14 cm, was located in a soundproof room with dimly lit. The maze was divided into four quadrants, a black platform (8 cm in diameter and

10 cm in height) was centered in one quadrant of the pool and submerged 1 cm below the water surface. The pool wall was surrounded by a black curtain; the camera up to the top of the pool was used to record the escape latencies and the path length of each mouse.

Briefly, mice were retrained during the first 2 days to find a 10 cm diameter visible platform, which was placed in the center of the northwest quadrant in the tank. Next, they underwent 5 consecutive days of navigation tests to find the hidden platform submerged 1 cm below the water surface. Each mouse was subjected to 2 trials per day with each trial lasting a maximum of 90 s. The animals failed to find the platform were gently guided to the platform and stay on it for 30 s to remember the location of the platform, and then returned to the cage. The escape latencies for escaping to the platform and the speed of each trial were recorded. On the 30th day's probe trial, the platform was removed and the mice allowed swimming freely within 120 s. The time spent swimming in the quadrant which located the platform before were measured for each mouse.

2.6. Brain tissue preparation

On the 30th day after behavioral tests, the mice were sacrificed by decapitation and the brains were removed for biochemical analysis. Two entire brains in each group were used for histological assessment and the hippocampus and cerebral cortex were separately dissected from the other brains, weighed, frozen in a refrigerator at −80 °C until used. Before detection, each part of the brain tissue was rapidly homogenized in ice-cold saline and the homogenates were centrifuged at 3500 rpm at 4 °C for 15 min. The supernatant was collected for assay.

2.7. Measurement of ACh, ChAT, AChE and M1 receptors

The levels of ACh and the activities of ChAT, AChE in the supernatant were measured using commercial assay kits (Nanjing Jiancheng) according to the manufacturer's directions with Synergy HT multifunctional microplate reader.

The levels of M1 receptors in the mouse brain were measured using an ELISA kit according to the manufacturers' protocols. The absorbance of treated supernatant was measured by ELISA to know its concentration indirectly and concentration of each sample was recorded.

2.8. Histopathological examination

For histopathology, the entire brains soak in 10% paraformaldehyde (PFA) solution for 48 h, and then transferred to 30% sucrose in the 0.1 mol/L PBS (pH 7.4) for 18 h until they sank for cryoprotection. The tissues were then kept in the final sucrose solution until sectioning. The brains were sliced into 5 μm with a section cutter then stained with hematoxylin–eosinreagent and then dehydrated with graded alcohol and mounted with neutral balsam medium to observe changes in the cortical and hippocampus neurons.

2.9. Statistical analysis

Results are expressed as the mean ± S.E.M. (n = 10). Data were analyzed by one-way analysis of variance (ANOVA) followed by Turkey post hoc test; * $p < 0.05$ was considered significant. Statistical analyses were performed using the SPSS software, version 19.0.

3. Results

3.1. Effect of ASHP on Y-maze test

In Y-maze test, there was no significant difference among all the groups on the numbers of arm entries (Fig. 2A). Scopolamine significantly decreased the spontaneous alternation behavior of mice compared with the control group (** $p < 0.01$; Fig. 2B). However, this decreased spontaneous alternation behavior induced by scopolamine was significantly relieved by donepezil and ASHP (# $p < 0.05$, ## $p < 0.01$; Fig. 2B).

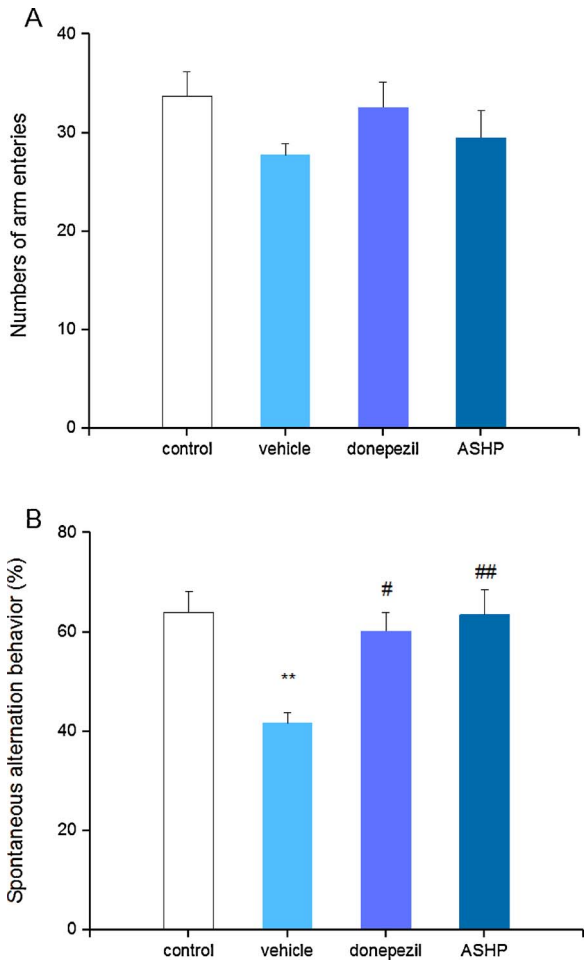

Fig. 2. Effect of ASHP on scopolamine–induced learning and memory impairment in the Y – maze (n = 10). The number of arm entries (A) and spontaneous alternation behavior (B) during an 8 – min session were measured. Date represent means ± S.E.M (** $p < 0.01$ versus control group; # $p < 0.05$, ## $p < 0.01$ versus vehicle group).

3.2. Effects of STB on morris water maze test

In Morris water maze test, there was no significant difference among all the groups in the initial visible platform tests (Fig. 3A). In the following 5 days of hidden platform tests, the vehicle group took longer time to reach the platform compared with the control group (*** $p < 0.001$; Fig. 3B). ASHP and donepezil treatment both significantly ameliorated the effect of scopolamine on escape latency and escape distance (## $p < 0.01$, ### $p < 0.001$; Fig. 3B). During the spatial probe test on the 30th day, the mice in control group, donepezil treated group and ASHP treated group spent much more time in the target quadrant (# $p < 0.05$; Fig. 3C). The number of crossing the platform of ASHP and donepezil treated group were closer to that in control group compared with vehicle group (# $p < 0.05$; Fig. 3D). Moreover, there is no significant difference in speed among groups (Fig. 3E).

3.3. Effect of ASHP on the level of ACh, M1 receptors and the activities of ChAT and AChE

The level of ACh and M1 receptors in the vehicle group was decreased significantly in hippocampus and cortex in comparison with the control group (** $p < 0.01$, *** $p < 0.001$; Fig. 4A–B). However,

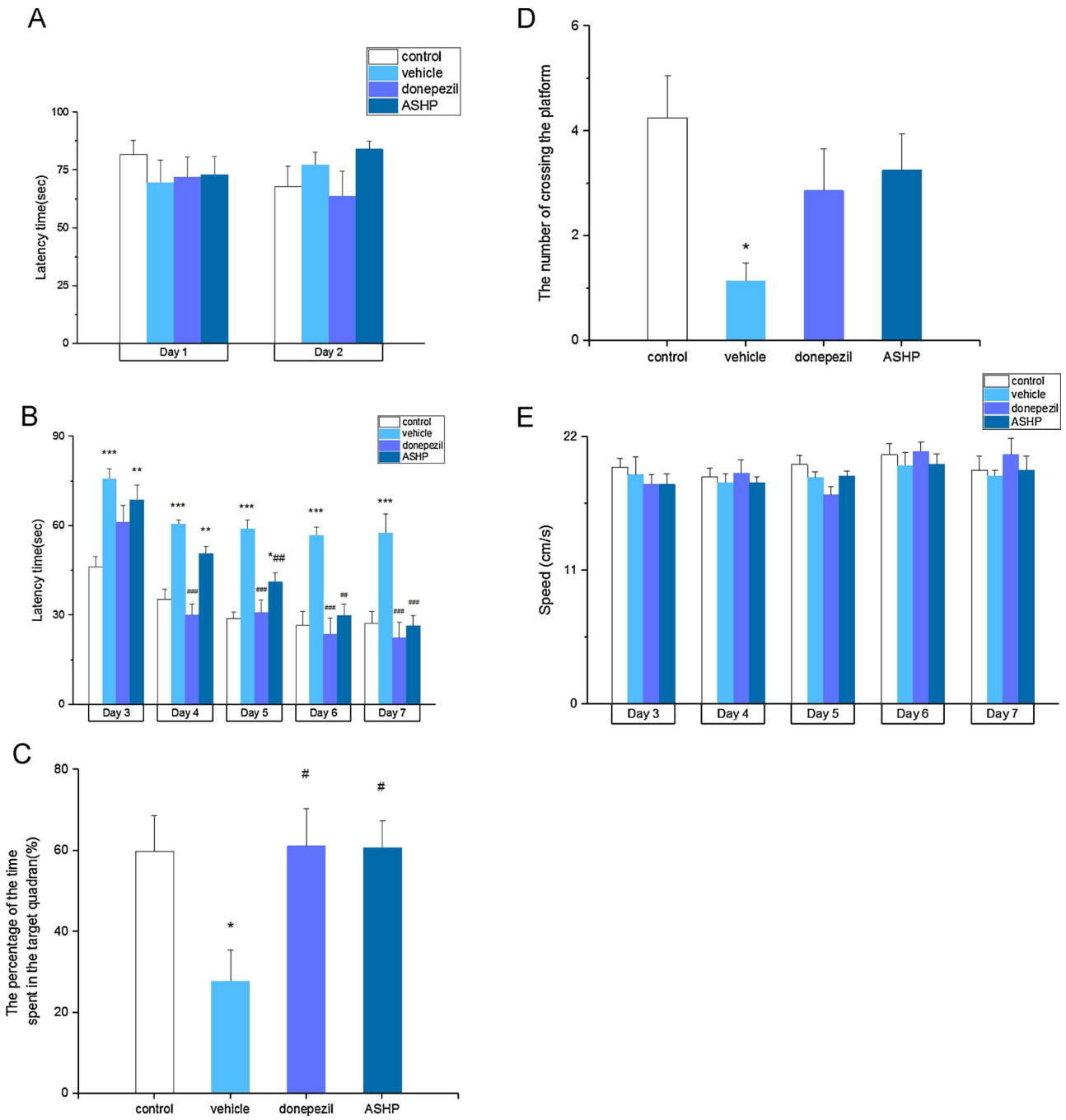

Fig. 3. Effect of ASHP on scopolamine – induced long – term memory and spatial learning impairment in the Morris water maze (n = 10). The latency time in the visible platform test was measured (A) . In training days escape latency was measured (B) and in the probe trai l the percents of total time (C) in the previous target quadrant and the numbers of crossing the platform (D) and the speed of all groups (E) are shown . Date represent means ± S.E.M (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus vehicle group).

treatment with ASHP ameliorated this scopolamine – induced decrease, even better than donepezil (# $p < 0.05$, ## $p < 0.01$; Fig. 4A–B). Comparison between the control group and the vehicle group, scopolamine induced a significantly increase in AChE activity in hippocampus and cortex (*** $p < 0.001$; Fig. 4C). ASHP conspicuously decreased the activity of AChE both in hippocampus and cortex, while donepezil conspicuously decreased the AChE activity, only in hippocampus, not in the cortex (## $p < 0.01$, ### $p < 0.001$; Fig. 4C). In addition, among the groups, there was no significant difference in the activity of ChAT (Fig. 4D).

3.4. Results of histological examinations

The neurons of the vehicle group in the CA1 region and cortex have shown a marked changing and a distinct difference in neuronal structure. Scopolamine induced nuclear pyknosis, karyolysis in the CA1 region and cortex (Fig. 5B). The group which ASHP was administered showed similar characteristic to the control group, even better than the donepezil group (Fig. 5C–D).

4. Discussion

As a key regulator of cognition, cholinergic neurotransmitter system

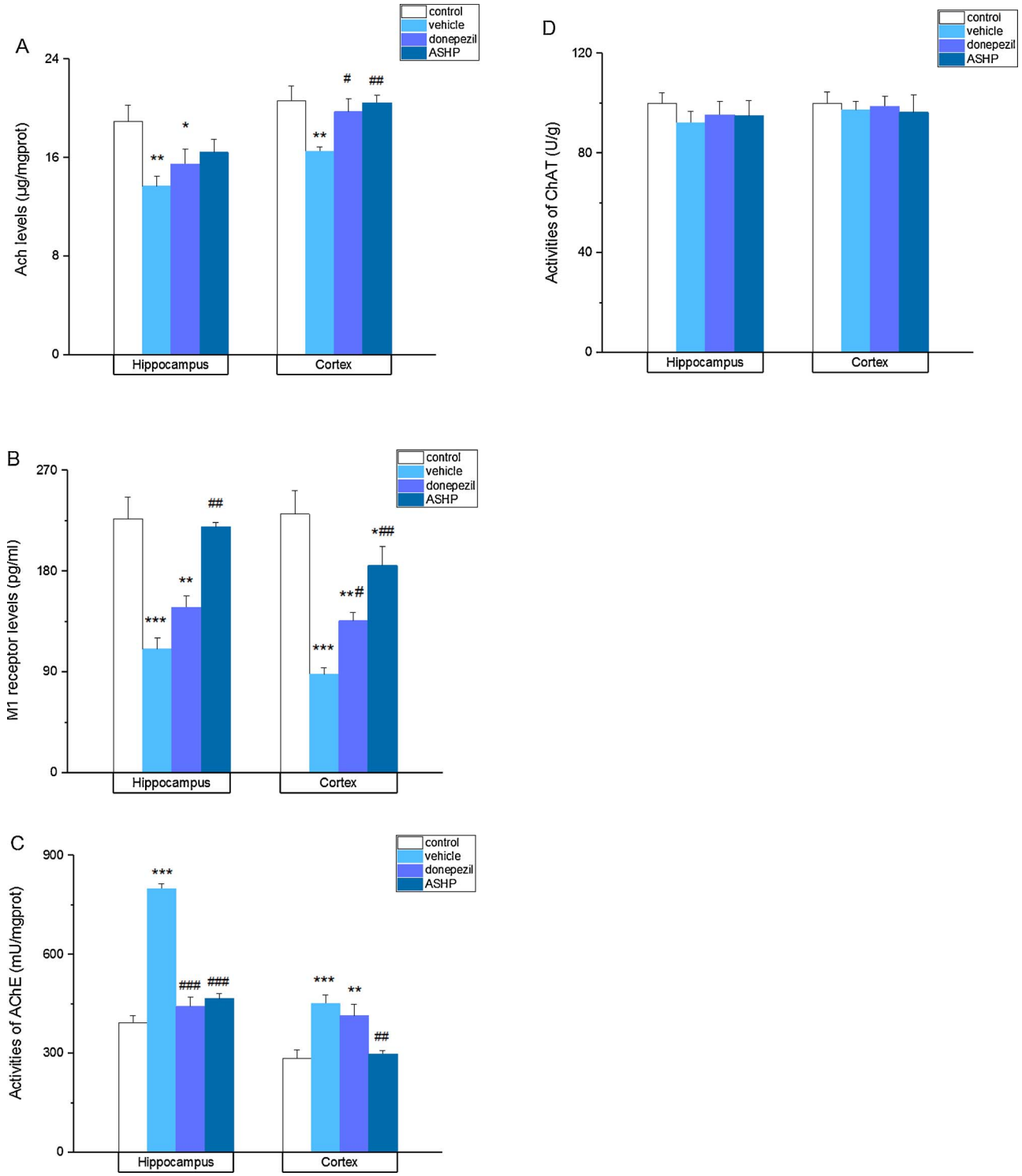

Fig. 4. Effects of ASHP on the level of ACh (A), M1 receptor (B) and the activities of AChE (C), ChAT (D) in hippocampus and cortex (n = 8) were shown. Values were mean ± S.E.M. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus vehicle group).

is a target of AD and has been extensively studied. Since there are some studies reported cholinergic neurons losses in the late stage AD brains but not in the earlier stages, many scientists disbelieve that cholinergic system damage is one of the pathogenic factors of AD [28–30]. However, cholinergic neurons loss did not happen doesn't mean no functional damaged. With the development of the research, more and more studies show that cholinergic dysfunction contributes to the early deficits in memory, according to the observation of aging and AD [31,32]. For example, basal nucleus of Meynert (bnM) is a major source of cortical cholinergic innervations, it had been reported cytoskeletal alterations in neurons of the bnM are already presented in the preclinical period of AD, it is possible that the reduction of cortical cholinergic activity may also be an early event in AD [31]. As a matter of fact, 3.5-month-old APPswe/PS1dE9 mice exhibited spatial memory impairment, accompanied by significantly decreased ACh and ChAT, evidence that cholinergic dysfunction may be early pathological of AD [33]. Changing AChE activity and M1 receptor level also play an important role in the memory loss and cognitive impairments [32,34].

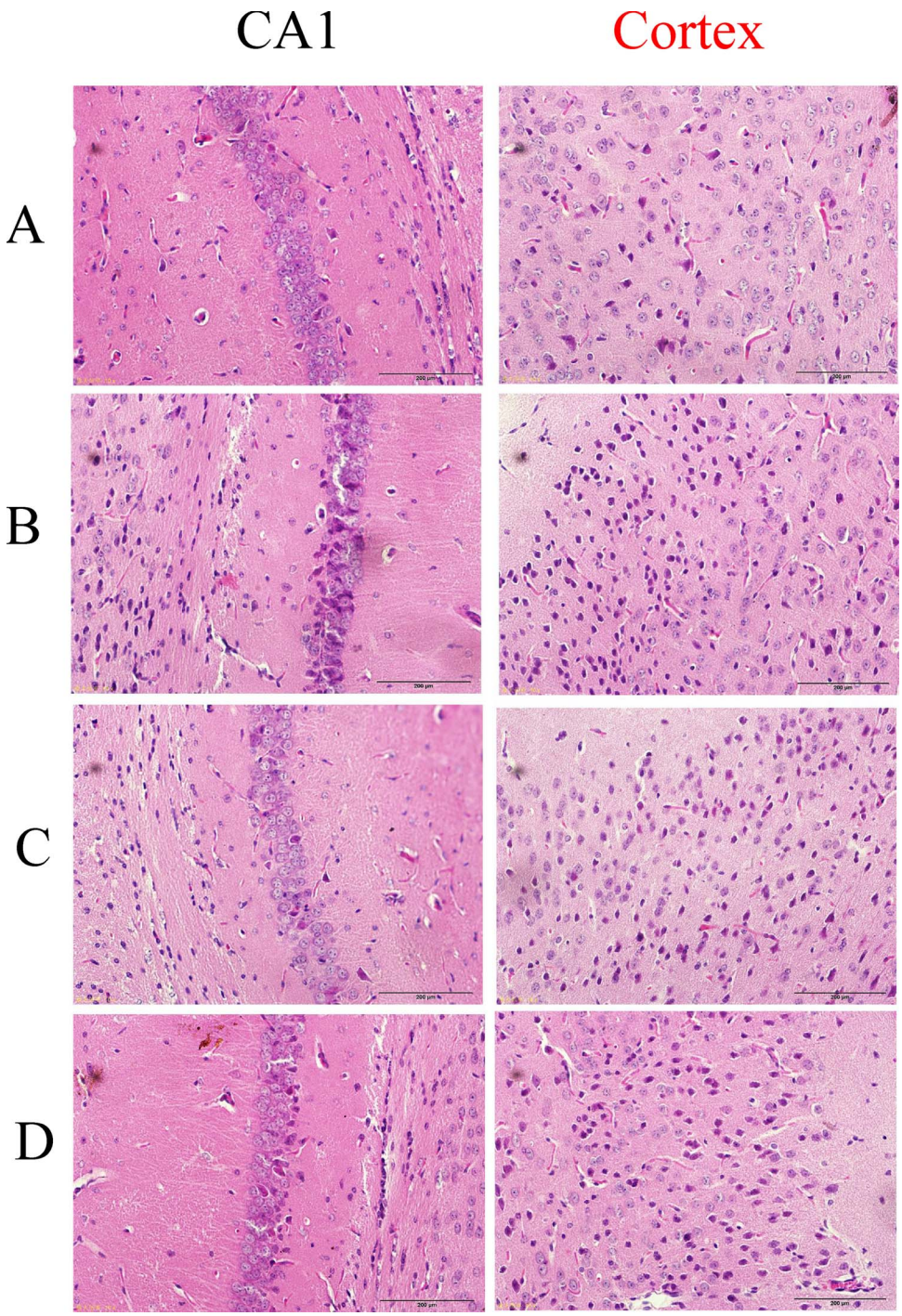

Fig. 5. Effect of ASHP on the histopathological changes in the CA1 region and cortex. Control group (A); vehicle group (B); donepezil group (C); ASHP group (D). The magnification was 400×.

As a tropane alkaloid drug, scopolamine was used as a muscarinic receptor antagonist, and it can induce anterograde memory impairment, particularly short-term memory and learning acquisition. So in this paper we used scopolamine – induced AD model [35]. And we observed that scopolamine can reduce the spontaneous alternation behavior in Y maze test (Fig. 2B); increase the escape latency (Fig. 3B), decrease the time spent in the target quadrant (Fig. 3C) and the number of crossing the platform (Fig. 3D) in Morris water maze test that means the model is well established.

As traditional Chinese medicine, *Alpinia oxyphylla* and *Schisandra chinensis* are both can be used as health products. According to previous studies, we put them together to probe into the effects of this herb pair on AD. In the paper, we used donepezil, a classical AChEI approved by FDA, to test the effect of ASHP treatment in behavior test. We found that ASHP can significantly ameliorate the cognitive impairment induced by scopolamine which is similar to donepezil. The results of Y maze and Morris water maze demonstrated this conclusion.

The results indicated that ASHP can increase the level of ACh (Fig. 4A), and decrease the activity of AChE (Fig. 4C), but there was no significant change for ChAT (Fig. 4D). So, we can speculate that ASHP may increase the levels of ACh by inhibiting the AChE activity, rather than regulating ChAT activity. In addition, it has been reported that AChE is linked to APP, the precursor of the Aβ peptide. Alteration of AChE activity increasing cholinergic deficit or exacerbating Aβ fibril formation and toxicity [36], if Aβ binds to AChE forming fibrils it will be more toxic than aggregates of Aβ alone [37,38]. So ASHP decreases

the activity of AChE to improve cognitive may through a variety of pathological pathways.

ACh can bind with nAChRs and mAChRs. The nAChRs are widely distributed throughout the central nervous system (CNS), however, among all subtypes, the α7 and α4, β2 subtypes are implicated in AD [39]. As GPCRs, mAChRs classified into five subtypes, M1–M5, and they mediate the effects of ACh in the CNS. Among all subtypes, M1 mainly distributed in cerebral cortex, hippocampus and neo striatum, and relate to cognition [40]. Since M1 receptors activation alters β-secretase (BACE1) levels via uncertain mechanisms, and activating α-secretase by PKC and MAPKs (such as ERK1/2), when M1 receptors stimulate by agonist or ACh, it can enhance sAPPα generation and reduces Aβ production. Although Aβ induces neurotoxicity by destabilizing β-catenin, M1 receptors stimulation counteracts that through the Wnt signaling pathway and inactivates GSK-3β via PKC activation, then improves neuronal survival. Moreover, activation of M1 receptors can inhibit tau phosphorylation, protect cells against apoptotic factors [41]. In this paper, we found that ASHP can increase the levels of M1 receptors (Fig. 4B) which reduced by scopolamine. The result of histological examinations also indicates that ASHP may have neuroprotective effects (Fig. 5), which proved our conjecture. However, the mechanism by which ASHP exerts neuroprotective effects is unclear, this will be our next research content.

In summary, we have demonstrated that ASHP could ameliorate memory performance in the behavior tests and improve cognitive impairments in a mouse model of AD induced by scopolamine. In addition, ASHP could increase the level of ACh and M1 receptors; decrease the activity of AChE, but not significantly affected the activity of ChAT. We also found ASHP may have neuroprotective effects. Since ACh, AChE and M1 receptors all relate to Aβ pathological mechanism, furthermore, previous studies shown not only *Alpinia oxyphylla* and *Schisandra chinensis,* but also their extracts have effects in Aβ1–42 mouse model, and produces neuroprotective effects [26–29], so we speculate that ASHP may interfere with Aβ pathological mechanism, and then play neuroprotective effects. Herb pair is characterized by multi-target and multiple pathways, ASHP may affect AD through a variety of pathways, so in the following research, we will study the role of ASHP in other AD related pathways and how it plays neuroprotective effects. Overall, the current data suggested that ASHP may serve as a novel agent for the prevention and treatment of AD.

Acknowledgements

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