Beneficial effects of asiaticoside on cognitive deficits in senescence-accelerated mice

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Abstract

The effect of asiaticoside isolated from Hydrocotyle sibthorpioides (AHS) on the promotion of cognition in senescence-accelerated mice (SAMP) was evaluated. Six-month old male SAMP8 mice were orally administered 20, 40 or 80 mg/kg AHS daily for three months. SAMR1 mice were used as a “normal aging” control. The results showed that treatment with AHS significantly improved learning and memory abilities in behavioral tests. AHS-treated mice showed higher antioxidant enzyme activity and lower lipid oxidation in serum compared with untreated SAMP8 mice. Mechanistically, studies showed that AHS markedly reduced the content and deposition of β-amyloid peptide (Aβ) by inhibiting the expression of mRNA for amyloid protein precursor, β-site amyloid cleaving enzyme-1 and cathepsin B and promoting the expression of mRNA for neprilysin and insulin degrading enzyme. In addition, AHS significantly increased the expression of plasticity-related proteins including postsynaptic density-95, phosphor-N-methyl-D-aspartate receptor 1, phosphor-calcium–calmodulin dependent kinase II, phosphor-protein kinase A Catalytic subunit, protein kinase Cγ subunit, phosphor-CREB and brain derived neurotrophic factor. Furthermore, AHS increased the levels of acetylcholine (Ach), but decreased cholinesterase (AchE) activity. These results demonstrated that AHS administration may prevent spatial learning and memory decline by scavenging free radicals, up-regulating the activity of antioxidant enzymes, decreasing the level of Aβ, ameliorating dysfunction in synaptic plasticity, and reversing abnormal changes in Ach level and AchE activity. Thus, AHS should be developed as a new drug to prevent age-related cognitive deficits.

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1. Introduction

Alzheimer’s disease (AD), one of the most prevalent age-dependent neurodegenerative disorders, is characterized by mild cognitive impairment at its onset followed by irreversible neuronal degeneration and dementia in later stages [1]. Approximately 5% of the population aged 65 and over are estimated to be affected by AD [2]. This projection certainly indicates a problem of considerable magnitude, particularly in terms of the number of patients suffering, the affected relatives and the negative socioeconomic outcomes of AD. Impairment of short-term memory, due to neuronal dysfunction and degeneration in the hippocampus and amygdale, marks the first stage in disease progression. Neuropathological hallmarks of AD include the deposition of β-amyloid (Aβ1–42)-containing senile plaques and the presence of intracellular neurofibrillary tangles in hippocampal and cerebral cortical regions [3,4]. The pathogenic mechanisms underlying AD include impaired cholinergic function, increased oxidative stress, induction of the amyloid cascade (i.e., Aβ deposition and plaque formation), deficiencies in steroid hormones, and the appearance of glutamate-mediated
excitotoxicity [2]. Of all these mechanism, the amyloid cascade hypothesis, which proposes a pivotal role for Aβ in the pathogenesis of AD, is the most widely accepted by investigators in this field [5]. Although the currently available drugs for dementia, such as acetylcholinesterase inhibitors, provide effective temporary treatment of memory dysfunction, they do not prevent or reverse the underlying neurodegeneration [6]. Research has shown that deterioration of memory begins prior to the onset of old age in animals, including humans [7]. Thus, it is extremely important to identify treatments that can prevent or retard AD-related memory decline and to explore preventive mechanisms to delay the onset of memory deterioration [8].

Herbal medicines have been used to treat AD, and many are now being collected and examined in an attempt to identify possible sources of anti-AD therapeutics [9]. Natural compounds, because of their structural diversity, provide a good opportunity to screen for anti-AD agents. Asiaticoside, a pentacyclic triterpenoid saponin, has been described to have antiulcer, antioxidant and anti-inflammatory activities [10]. Asiaticoside also offers protection against chemical-induced hepatotoxicity [11]. Some studies demonstrated that treatment with asiaticoside might induce antidepressant-like effects [12], and attenuate neurotoxicity induced by 1-methyl-4-phenyl-1,2,3, 6-tetrahydroprpyidine (MPTP) in a rat model of Parkinsonism [13]. Moreover, recent studies indicated that asiaticoside has shown to rescue B103 rat neuroblastoma cells against Aβ25–35 and H2O2-induced neurotoxicity [14]. Based on these reports, it would be of great interest to determine the effects of asiaticoside on cognitive deficits. In the present study, we investigated the effect of administering asiaticoside isolated from *Hydrocotyle sibthorpioides* (AHS) by oral gavage for 3 months on learning and spatial memory loss in SAMP8 mice. The molecular mechanisms involved in the prevention of learning and spatial memory loss were also studied.

Cholinesterase inhibitors are most widely used to treat AD. Their effect is to ameliorate symptoms without achieving permanent improvement. Huperzine A (9-amino-13-ethylidene-11-methyl-4-azatricyclo[7.3.1.0(3,8)]trideca-3(8),6,11-trien-5-one) is a lycopodium alkaloid isolated from the moss *Huperzia serrate* and acts as a potent, highly specific and reversible inhibitor of acetylcholinesterase, with a better therapeutic index than physostigmine and tacrine [15]. In this study, huperzine A was used as positive control.

### 2. Materials and methods

#### 2.1. Drugs and chemicals

*H. sibthorpioides* was purchased from Nanning Qianjinzi Chinese Pharmaceutical Co. Ltd (Nanning, China). Voucher specimen (HSL2011091327) was identified by Q.F. Huang in the First Affiliated Hospital of Guangxi Traditional Chinese Medicine University and deposited in the herbarium of the Department of Pharmacology of Guangxi Medical University.

Huperzine A was purchased from Yuzhong Drug Manufacturing (Henan, China). Malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GSH-PX) kits were obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China).

2.2. Preparation of asiaticoside from *H. sibthorpioides* (AHS)

The powder of dried plant of *H. sibthorpioides* (10 kg) was extracted with 80% ethanol by filtration. The solvent was evaporated under a vacuum to obtain 893.7 g crude extract, which was extracted with petroleum ether, CHCl3 and water-saturated n-butanol successively. The water-saturated n-butanol (367.2 g) was dissolved in MeOH and filtered through a syringe filter (0.45 μm). The filtrate yielded a yellow powder after concentration, which was purified by recrystallization in MeOH to yield the crude saponin (195.4 g). The crude saponin was then subjected to chromatography on a silica gel column (200–300 mesh, Yantai, PR China; Ø10 cm × 300 cm) eluting with a gradient mixture of CHCl3 and MeOH (0–100% MeOH, 2500 ml each fraction). The eighth fraction yielded a white crystal after concentration, which was purified by Sephadex LH-20 and preparative HPLC to produce compound (81.6 g). Its structure was elucidated on the basis of physicochemical properties and spectral data: mp 231–233 °C; IR (KBr, ν/cm⁻¹) 6: 3416, 2927, 1373, 1638, 1456, 1379, 1270, 1061, 961; ESI-MS m/z: 981.5 [M + Na]⁺; ¹H NMR (300 MHz, DMSO-d₆) 6: 0.85, 0.89, 1.05, 1.39, 1.07, 1.10, 1.17; ¹³C NMR (CDCl₃, 100 MHz) δ: 125.6, 138.1, 140.175.9, 104.8, 102.4, 95.3. The results showed that the compound is asiaticoside, with its molecular formula and molecular weight being C₆₂H₈₀O₁₉ and 959.12, respectively. Its chemical structure was shown in Fig. 1. The compound was normally stored at 4 °C. It was dissolved in distilled water and diluted with physiologic saline for test in animal.

2.3. Animals and treatment

Six-month-old male senescence-accelerated mouse prone/8 (SAMP8) and senescence-accelerated mouse resistant/1 (SAMR1) were intraperitoneally injected with 10 kg/kg body weight of test, vehicle or positive control in a randomized block design (8 mice/treatment). Animals were housed at 25 ± 2 °C, 50 ± 10% humidity, and 12 h light/dark cycle. The light period began at 8:00 AM. The weight loss of animals was monitored weekly. Treatment with asiaticoside had no obvious body weight effects. The animals were divided into 4 groups based on their body weight. Each group received vehicle, asiaticoside (10 mg/kg), huperzine A (10 mg/kg) or a donepezil (10 mg/kg) for 3 months, once a day.
(SAMR1) mice, weighing 25 ± 2.0 g, were provided by the First Affiliated Hospital of Tianjin Traditional Chinese College. The research was conducted according to protocols approved by the institutional ethical committee of Guangxi Medical University (approval no.: 20110501202). All mice were housed under controlled conditions at 25 ± 2 °C, with a relative humidity of 60 ± 10%, room air changes 12–18 times/h, and a 12-h light/dark cycle. Feed and water were made available ad libitum.

Ninety mice (15 SAMR1 normal control mice and 75 SAMP8 mice) were used in the study. All mice were allowed 1 week to adapt to their environment before being used for experiments. The SAMP8 mice were then divided into 5 groups with 15 animals per group. Group I was the SAMR1 control, while Group II was the SAMP8 control. These two groups were given an equivalent amount of normal saline. Group III was the positive control; huperzine (0.02 mg/kg/day) was administered orally to the mice. Groups IV–VI were the AHS-treated groups; AHS was administered intragastrically to mice in these groups at 20, 40 and 80 mg/kg/day, respectively. The total duration of the experiment was 3 months.

Behavioral tests were assessed in all mice at the end of drug administration. After the behavioral test, the mice were euthanized by decapitation. At euthanasia, blood was sampled from the carotid artery, centrifuged at 3000 rpm for 10 min at 4 °C and the serum was collected. Immediately after decapitation, the brain was rapidly excised and dissected on ice to obtain the hippocampus and the cerebral cortex. All of the serum and tissue samples were stored at −80 °C until analysis.

2.4. Morris water-maze task

After the open field test, the Morris water-maze task was selected to evaluate spatial learning and memory. The water maze was a circular pool 90 cm in diameter and 50 cm in height with a black inner surface. The tank was placed in a dimly lit soundproof room with several visual cues. The pool was divided into 4 quadrants of equal area, and filled to a depth of 30 cm with water at 20–21 °C. A black platform 6 cm in diameter and 1 cm below the surface of the water was placed in one of the quadrants. For memory acquisition (training), each mouse underwent four successive trials a day for 6 days. The interval between trials every day was 15 min to let mice recover physical capacity. The sequence of water-entering points differed each day, but the location of the platform was constant. Latency to find the platform was measured up to a maximum of 60 s. On locating the platform, the mouse was left there for 10 s prior to the next trial. If the mouse failed to locate the platform within 60 s, it was guided to the platform and allowed to stay there for 10 s. Latency and average speed to reach the platform were recorded for each trial.

The day after the last training session, mice were subjected to a probe trial session in which the platform was removed from the pool. The mice were allowed to swim for 60 s to search for it. Latency (the first time that the mice crossed the site of the removed platform), searching frequency (the number of times that the mice crossed the site of the removed platform), and the swimming time within the target quadrant were recorded and analyzed.

2.5. Passive avoidance test

The apparatus (40 cm long, 20 cm wide and 30 cm high) consisted of an illuminated chamber connected to a dark chamber by a guillotine door. Electric shocks were delivered to the grid floor by an isolated stimulator. Animals started the test in the light compartment. After a brief orientation (10 s), the gate was opened to allow the mouse to enter the darkroom. Once the mouse was inside the darkroom, the gate was closed and 0.5 mV, 1 s shocks (repeated three times with an interval of 5 s) were executed. The time that the mouse stayed in the light room was recorded. Each mouse was exposed to the test on the first, second, third and seventh day of the experiment. After shock administration, the time that the mouse hesitated before entering the darkroom again was observed. Each test was performed for no longer than 180 s.

2.6. Determination of levels of MDA, NO, SOD, GSH-PX and GSH in the brain

Brain levels of MDA, NO, SOD, GSH-PX and GSH were measured using commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer’s instructions.

2.7. Measurement of Aβ1-42 protein and plasticity-related proteins in the hippocampus by Western blotting

The mouse hippocampi were lysed in a buffer containing 50 mM Tris–HCl (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate, and 5% β-mercaptoethanol. Protein lysate (50 μg) was separated by 8% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking in a 5% nonfat dry milk solution in washing buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween-20, the membranes were incubated overnight at 4 °C with different antibodies: rabbit polyclonal anti-β amyloid 1–42 (Abcam, UK, 1:500), rabbit monoclonal anti-PSD-95 (Thermo, USA, 1:200), rabbit polyclonal anti-phospho-NMDAR1 (Ser896) (Invitrogen, USA, 1:1000), and rabbit polyclonal anti-phospho-CAMKII (Thr286) (Upstate Biotechnology, USA, 1:1000), rabbit polyclonal anti-phospho-PKA Catalytic subunit (Ser38) (Millipore, USA, 1:1000), mouse monoclonal anti-PKCγ (Invitrogen, USA, 1:200), mouse monoclonal anti-phospho-CREB (Ser133) (Cell Signaling Technology, USA, 1:1000), and anti-BDNF (Roche Molecular Biochemicals, Germany, 1:500). After washing three times with phosphate buffer solution, the membranes were incubated for 1.5 h with horseradish peroxidase-conjugated secondary antibody at room temperature. Following a final washing in phosphate buffer solution, the antigen-antibody staining was visualized using diaminobenzidine. Finally, the sections were washed in distilled water, dehydrated through an ethanol series, cleared with xylene and mounted in gel. The band was then scanned, and the intensity of the protein was measured using densitometry software. β-actin was used as the internal control. All values were normalized to β-actin and expressed as arbitrary units relative to the control.
2.8. Preparation of total RNA and cDNA

Total RNA was extracted from the hippocampus using Trizol reagent (Invitrogen) according to the manufacturer’s instructions and stored at −70 °C. A ratio of A260/A280 in the range of 1.9–2.0 was used. The RNA concentrations were determined from the absorbances at 260 nm. Denaturing formaldehyde 1% agarose gel electrophoresis was used to check the integrity of the total RNA. cDNA was synthesized from total RNA by reverse transcriptase (Fermentas) with the primer oligo (dT) 12–18 and was stored at −20 °C.

2.9. Relative quantitative real-time PCR

By using the primer sequences listed in Table 1, relative quantitative real-time PCR analysis was performed with an Applied Biosystems Prism 7500 Fast Sequence Detection System. The mRNA level of amyloid protein precursor (APP), β-site amyloid cleaving enzyme-1 (BACE1), cathepsin B (CatB), neprilysin (NEP) and insulin degrading enzyme (IDE) in the hippocampus was analyzed. Mouse GAPDH gene was used as an endogenous control.

SYBR® Green Real-time PCR Master Mix (TOYOBO) and iTaq™ SYBR Green Supermix with Rox (BIO-RAD) were used according to the manufacturer’s instructions. The thermal cycler conditions were as follows: hold for 120 s at 95 °C, followed by three-step PCR for 40 cycles of 95 °C for 15 s, 58 °C (GAPDH, APP, BACE1 and CatB) or 60 °C (GAPDH, NEP and IDE) for 30 s, and 72 °C for 45 s. Levels of mRNA expression were determined using the 7500 Fast Real-Time PCR System SDS software version 2.0.5 (Applied Biosystems) according to the ΔΔCT method.

2.10. Assay of Ach

Ach was measured using the method of Zhong et al. [16]. Briefly, 0.8 ml of brain homogenate (10%, w/v) was mixed with 1.4 ml of pure water followed by addition of calabarine sulfate. Then, 0.8 ml of trichloroacetic acid (1.84 M) was added and mixed. The mixture was centrifuged at 3000 rpm for 15 min, and the supernatant was removed and centrifuged at 3000 rpm for 10 min before the final supernatant was removed. Alkalinity hydroxyamine (1 ml) was added to 1.0 ml of the final supernatant and mixed. Subsequently, 0.5 ml of 4 M HCl and 0.5 ml of 0.37 M ferric chloride were added, and the mixture was shaken violently. The absorbance at 540 nm was measured. Ach levels were expressed as μg per mg of protein.

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td>5′-TTCGCAACTGATTCCAGGATTT-3′</td>
<td>5′-TCATGACATACCGGTTGCTA-3′</td>
</tr>
<tr>
<td>BACE1</td>
<td>5′-AGGCGCTCCACCTGAGAC-3′</td>
<td>5′-GCCTACTATGGACCGAATA-3′</td>
</tr>
<tr>
<td>CatB</td>
<td>5′-CAAGAATCTTCCTCCACCC-3′</td>
<td>5′-TGTCCTACGACCCAAGC-3′</td>
</tr>
<tr>
<td>NEP</td>
<td>5′-TCTTGAACGATCCGACGATCC-3′</td>
<td>5′-CTCCGACAGCAGTTCTCAT-3′</td>
</tr>
<tr>
<td>IDE</td>
<td>5′-TGTCATCAATTTGGGCGAG-3′</td>
<td>5′-AACCTCCGCGTCTCTCCT-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GAPDH</td>
<td>5′-CAAGTGTTGTCACTCC-3′</td>
</tr>
</tbody>
</table>

### Notes

AchE activity in the supernatant of brain homogenate was measured according to the method of Zhong et al. [16]. Briefly, a reaction mixture that contained 470 μl of sodium phosphate (1 mM, pH 8.0), 167 μl of 2% DTNB and 33 μl of homogenate was incubated for 5 min at 37 °C. Then, 280 μl of acetylcholine iodide (2 mM) was added to the reaction mixture. After incubation for 3 min at 37 °C, the reaction was terminated by adding 50 μl of neostigmine (4 mM). The absorbance was measured at 412 nm at room temperature. AchE activity was expressed as μmol/min/mg protein.

2.11. Determination of AchE activity

2.12. Statistical analysis

### 3. Results

#### 3.1. The effects of AHS on spatial learning and memory deficits

Mice were trained in the water maze for 6 days. SAMR1 mice rapidly learned the location of the platform, but SAMP8 mice had significantly longer escape latencies on every test day. Treating SAMP8 mice with 40 or 80 mg/kg AHS or 0.02 mg/kg huperzine A resulted in a significant decrease in escape latency (Fig. 2A).

In the probe test, latency (the first time that the mice crossed the site of the removed platform) was increased and searching frequency (the number of times that the mice crossed the site of the removed platform) was decreased in SAMP8 mice compared to SAMR1 mice. The latency was decreased by AHS or huperzine A administration, and searching frequency was increased significantly by administration of 40 and 80 mg/kg AHS. In addition, treatment with AHS or huperzine A increased the swimming time within the target quadrant (Fig. 2B).

#### 3.2. Passive avoidance test

As shown in Fig. 3, the passive avoidance time in the SAMR8 control group was significantly shorter than in the SAMR1 control group. However, the passive avoidance times

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3.3. The effects of AHS on brain levels of MDA, NO, SOD, GSH and GSH-PX

Brain levels of MDA, NO, SOD, GSH and GSH-PX were detected. As shown in Fig. 4, MDA and NO contents were significantly higher in SAMP8 mice when compared with SAMR1 mice. Administration of AHS at 40 and 80 mg/kg significantly decreased the MDA and NO contents (Fig. 4A). In addition, the SOD, GSH and GSH-PX activities were significantly lower in the SAMP8 mice when compared with the SAMR1 mice. However, the SAMP8 mice treated with AHS showed a significant improvement towards the SAMR1 control value (Fig. 4B).

3.4. The effects of AHS on Aβ1–42 protein in the hippocampus

The expression of hippocampal Aβ1–42 protein in the SAMP8 control group was significantly higher when compared with its expression in the SAMR1 control group. This up-regulation was inhibited after treatment with AHS or huperzine A (Fig. 5).

3.5. The effects of AHS on the expression of synapse plasticity-related proteins in the hippocampus

To evaluate the effects of AHS on synapse plasticity-related proteins in the hippocampus, we conducted an analysis of the expression of postsynaptic density-95 (PSD-95), phosphor-N-methyl-D-aspartate receptor 1 (p-NMDAR1), phospho-calcium–calmodulin dependent kinase II (p-CaMKII), phospho-protein kinase A Catalytic subunit (p-PKA C) and protein kinase Cy subunit (PKCy), phospho-CREB (p-CREB).

Fig. 2. The effects of AHS on spatial learning and memory deficits in the Morris water maze performance. (A) The training trial sessions. Figure panel A lists the data of the latency time during the training trial sessions of the Morris water maze performance. Spatial learning and memory ability of SAMP8 was impaired all throughout the test; and the impairment was improved by the administration of different dosages AHS. (B) The probe trial session. Latency: the first time that the mice crossed the former platform; searching frequency: the number of times that the mice crossed the site of the removed platform; time in target area: the swimming time within the target quadrant. Data values are expressed as means ± SE, *P < 0.05 vs. SAMP8 control.

Fig. 3. The effects of AHS on the learning and memory impairment of SAMP8 mice in the passive avoidance test. Avoidance tests were performed for three consecutive days as well as on the seventh day of the study. Data values are expressed as means ± SE, *P < 0.05 vs. SAMP8 control.

Fig. 4. The effects of AHS on brain levels of MDA, NO, SOD, GSH-PX and GSH. Data values are expressed as means ± SE, *P < 0.05 vs. SAMP8 control.
and brain derived neurotrophic factor (BDNF). As shown in Fig. 6, significantly lower expression of these proteins was observed in the SAMP8 control group when compared with the SAMR1 control. SAMP8 animals treated with AHS exhibited a significant increase in the expression of these proteins.

3.6. Effects of AHS on the expression of genes related to Aβ

Statistical analysis showed higher APP, BACE1 and CatB levels and significantly lower NEP and IDE levels in the SAMP8 control group compared with the SAMR1 control group. Treatment with AHS induced changes in the mRNA levels: APP, BACE1 and CatB mRNA levels were decreased and NEP and IDE mRNA levels were markedly increased after treatment (Fig. 7).

3.7. Ach level and AchE activity in the mouse brain

As shown in Fig. 8, a significantly lower level of Ach was observed in the SAMP8 control group when compared with the SAMR1 control. Mice treated with AHS exhibited a significant increase in the level of this choline. In addition, AchE activity was significantly higher in the SAMP8 control group. However, this higher AchE activity was not observed in the brains of AHS-treated mice.

4. Discussion

Senescence-accelerated mouse prone 8 (SAMP8) is an autogenic senile strain of mouse characterized by age-related deteriorations, such as loss of memory and retention at an early age; these mice also produce increased amounts of amyloid precursor protein (APP) and Aβ characteristics that are similar to those observed in AD patients [17]. These features make SAMP8 an ideal model for studying the development and progression of AD-related cognitive deficits.

Fig. 5. The effects of AHS on Aβ1-42 protein in hippocampus in SAMP8 mice. The bands are a representative of the blot. Lane 1, SAMR1 control group; lane 2, SAMP8 control group; lane 3, 0.02 mg/kg huperzine A-treated group; and lanes 4-6, 20, 40 and 80 mg/kg AHS-treated groups. 50 μg protein lysate was separated by 8% SDS-PAGE in Western blotting. Relative protein level between the tested target protein and internal standard β-actin band was calculated and labeled on Y axis. Data values are expressed as means ± SE, *P < 0.05 vs. SAMP8 control.

Fig. 6. The effects of AHS on the expressions of synapse plasticity-related proteins in hippocampus. (A) The bands are a representative of the blot. Lane 1, SAMR1 control group; lane 2, SAMP8 control group; lane 3, 0.02 mg/kg huperzine A-treated group; lane 4-6, 20, 40 and 80 mg/kg AHS-treated groups. (B) Relative protein level between the tested target protein and internal standard β-actin band was calculated and labeled on Y axis. Data values are expressed as means ± SE, *P < 0.05 vs. SAMP8 control.

Fig. 7. APP, BACE1, CatB, NEP and IDE mRNA expression were assayed by real-time PCR in the hippocampus. APP: amyloid protein precursor; BACE1: β-site amyloid cleave enzyme-1; CatB: cathepsin B; NEP: neprilysin; IDE: insulin degrading enzyme. Data values are expressed as means ± SE, *P < 0.05 vs. SAMP8 control.

Fig. 8. The effects of AHS on Ach level and the AchE activity of mice brains. Data values are expressed as means ± SE, *P < 0.05 vs. SAMP8 control.
SAMP8 a good model to investigate the fundamental mechanisms of age-related learning and memory deficits and to evaluate the action of drugs [18,19]. SAMR1 mice, which are genetically related to SAMP8 but resistant to accelerated senescence, have always been used as the normal control [20].

In the present study, we found that SAMP8 mice developed a remarkable deficiency in the hippocampal-dependent Morris water maze task and a decline in passive avoidance in the passive avoidance test when compared with SAMR1 mice. Our behavioral results are consistent with previous results [21,22]. Furthermore, the present study is the first report showing that long-term AHS consumption prevents impaired learning and memory in SAMP8 mice. The efficacy of AHS is dose-dependent, with significant benefits at AHS concentrations of 40 and 80 mg/kg. Although AHS at 20 mg/kg consumption tended to increase memory, the effect was weak.

Free radicals become an active field in aging research because of their potential involvement in many degenerative processes and in many neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases [23]. These free radicals can be scavenged by endogenous antioxidants including GSH, SOD and GSH-PX. MDA is a by-product of lipid peroxidation induced by free radicals and is widely used as a biomarker of oxidative stress [24]. MDA can lead to injury of cellular membrane and impairment of the central nervous system [25]. NO is the intracellular and intercellular gas message molecule, also a strong gas molecule free radical. Under normal physiological conditions, NO play important roles in physiological function of the central nervous system and the main function of NO is to modulate the generation of memory and pain. But, NO has been reported to have neurotoxicity and induce cell apoptosis as well as sleep deprivation (SD) when expressed excessively [26]. In this study, we found that MDA and NO levels were higher and GSH, SOD and GSH-PX activities were lower in SAMP8 mice when compared with SAMR1 control. The converse was true after long-term administration of AHS intragastrically, and significantly elevated GSH, SOD and GSH-PX activities and markedly decreased MDA and NO levels were observed. This result indicates that AHS treatment significantly enhances the antioxidant defense systems and alleviates the harmful effects of free radicals in SAMP8 mice.

Aggregation and deposition of Aβ in the brain are key steps in the pathogenesis of AD that elicits a cascade of cellular events leading ultimately to neuronal loss and dementia [27]. We found that the content of Aβ in the hippocampus of SAMP8 was significantly higher than in the SAMR1 control. Long-term administration of AHS significantly decreased the Aβ content, and this decrease may be one of the mechanisms of action of AHS in ameliorating cognition degeneration in SAMP8 mice.

An imbalance between the production of Aβ and its removal causes Aβ accumulation. Aβ is produced by proteolytic processing of APP. Proteases referred to as β-secretase and γ-secretase cleave at the N- and C-termini of Aβ within APP to generate Aβ. Aβ then undergoes secretion to provide extracellular Aβ that produces neurotoxic effects, with aggregation and accumulation in amyloid plaques of AD brains. BACE1 and CatB are generally considered to be the critical β-secretases [28–30]. Additionally, several enzymes within the central nervous system are capable of degrading Aβ. Among these enzymes, there is increasing evidence that NEP and IDE are primarily responsible for degrading Aβ in the brain [31]. Therefore, five genes associated with the Aβ pathway, APP, BACE1, CatB, NEP and IDE, were analyzed to explore the molecular mechanism of the effect of AHS on abnormal Aβ levels. The results revealed that AHS could significantly decrease the levels of the former three genes and increase the levels of the latter two genes. This indicates that AHS may lower Aβ content by modulating the Aβ pathway (particularly inducing a decline in the levels of APP, BACE1 and CatB and a rise in the levels of NEP and IDE).

Initially, the decline of memory function with age is believed to be due to changes in synaptic function rather than a loss of neurons [32]. Postsynaptic density 95 (PSD-95), highly abundant at the postsynaptic density and a major constituent of dendritic spines, can be regarded as a key molecule in neuronal information storage-related processes [33]. The processes of learning, memory formation and functional plasticity, such as long-term potentiation (LTP), are associated with the phosphorylation state of N-methyl-D-aspartic acid receptors (NMDARs) and Ca2+/calmodulin-dependent protein kinase II (CaM KKII) [34]. NMDA receptor subunit 1 (NMDAR1) is a subunit required for functional receptor formation [35]. A previous study reported impaired learning and memory in rats in which the NMDAR1 subunit was knocked down in a subset of hippocampal neurons [36]. CaM KKII, acting through its autophosphorylation properties, is thought to represent a “molecular switch” for the transition from short-term to long-term information storage [37]. There is strong evidence that the protein kinase C (PKC) and protein kinase A (PKA) signaling pathways, especially those of isoenzyme-PKA Cβ and PKCγ, regulate important molecular events in learning and memory processes and in the neurodegenerative pathophysiology of AD [38]. In addition, cAMP response element binding protein (CREB) has been identified as a key molecule initiating the transcriptional activation of other genes encoding proteins that presumably play an important role in the structural and functional changes underlying information storage [39]. Indeed, CREB activation through its phosphorylation on Serine-133 (p-CREB) controls the induction of regulatory immediate-early genes whose products subsequently induce the transcription of late downstream genes and activate direct “effector” proteins, such as structural proteins, signaling enzymes or growth factors that are essential for long-term memory. Brain-derived neurotrophic factor (BDNF) is a powerful modulator of neuronal excitability and synaptic transmission and plays a role in hippocampal-dependent learning and memory [40,41]. In this study, the expression of the above mentioned synaptic plasticity related proteins was significantly lower in SAMP8 mice, but was elevated by 40 and 80 mg/kg AHS. These results suggest that the protective effect of AHS ensuring integrity of hippocampal synapses may be a neurobiological basis for improving cognition deterioration.

The cholinergic system has been implicated in several neuropsychic functions, such as learning, memory, sleep, etc. [42]. Ach plays a principal role in modulating these functions [43]; AchE is responsible for the degradation of Ach to acetate and choline in the synaptic cleft. Therefore, we analyzed the Ach level and the AchE activity in the brains of each group of mice. We observed that the AchE activity was significantly higher in SAMP8 mice. As a consequence of the increase of AchE activity, the Ach level was lower in SAMP8 mice. Long-term treatment with AHS reversed the abnormalities in Ach.
level and AChE activity, suggesting that this compound may improve dysfunction of the cholinergic system.

In addition, our results showed that AHS prevented spatial learning and memory decline like the positive control drug huperzine A. What’s surprising is that mice in the huperzine A-treated group exhibited typical symptoms such as reduced activity, poor appetite, sparse and coarse hair and significant weight loss in contrast to SAMR1 normal control group. However, mice administrated with AHS maintained good phenotype and ingestive behavior similar to normal mice (data not shown). This further substantiates the developing value of AHS in protection against cognitive deficits due to its equal contribution but reduced side effect compared with huperzine A.

In conclusion, our study demonstrated that AHS significantly improved cognitive deficit and neuropathologic lesions in SAMP8 mice, and these effects may be mediated by scavenging free radicals, modulating the activity of antioxidant enzymes, decreasing the content and deposition of Aβ, improving the dysfunction of synaptic plasticity, and reversing the abnormality of ACh level and AChE activity. These data suggest that AHS is helpful in preventing age-related cognitive deficits.

Conflicts of interest

The authors do not have any conflicts of interest to disclose.

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References


