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Coadministration of huperzine A and ligustrazine phosphate effectively reverses scopolamine-induced amnesia in rats

Jun Shi [a,c], Qingfei Liu [b,*], Yiming Wang [c], Guoan Luo [a,c,*]

[a] School of Pharmacy, East China University of Science and Technology, Shanghai 200237, PR China
[b] School of Medicine, Tsinghua University, Beijing 100084, PR China
[c] Department of Chemistry, Tsinghua University, Beijing 100084, PR China

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ABSTRACT

In the present study, whether coadministration of huperzine A (HA) and ligustrazine phosphate (LP) could effectively improve the memory deficits in association with ameliorating cholinergic impairment and oxidative stress in the scopolamine-induced amnesia rats was assessed. The effects of treatment with Coa [HA (0.14 mg/kg, i.g.) and LP (110 mg/kg, i.g.)] on amnesia were investigated in Morris water maze. Furthermore, the effects on the activities of acetylcholinesterase (AChE) and antioxidant enzymes within the cerebral cortex and hippocampus were evaluated, and the lipid peroxidation product malondialdehyde (MDA) was also analyzed. As a result, coadministration of HA and LP for 10 consecutive days could markedly reverse the scopolamine-induced learning and memory impairment determined by the Morris water maze test. Moreover, AChE activity was significantly inhibited, and superoxide dismutases (SOD) and glutathione peroxidase (GSH-Px) activities were significantly increased with a remarkable reduction in the level of MDA. In conclusion, coadministration of HA and LP effectively prevented cholinergic impairment and oxidative damage, thereby resulting in improvement of spatial learning memory in rats induced by scopolamine. The results suggested that coadministration of HA and LP might offer a novel poly-therapeutic drug regimen for preventing Alzheimer's disease (AD).

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease with piecemeal loss in memory, which mostly affects the elderly population (Brookmeyer et al., 2007). The pathophysiology of AD is complex including the deposition of the senile plaques mainly composed of amyloid beta protein (Aβ), neurofibrillary tangles in patients' brain (Armstrong, 2006; Portelius et al., 2006), abnormalities of cholinergic, glutamatergic, adrenergic, serotonergic and dopaminergic neurotransmission, and the potential involvement of oxidative stress and inflammatory (Cummings, 2004). Cholinergic hypofunction may be directly related to the severity of cognitive dysfunction and memory loss in AD patients (Schliebs and Arendt, 2006). Currently, the most successful approach for AD treatment is to inactivate acetylcholinesterase (AChE). The AChE inhibitors, such as tacrine, donepezil and rivastigmine, can increase the availability of acetylcholine (ACh) at cholinergic synapses, slow the progression of dementia symptoms and enhance cognitive process in animals and humans (Davidsson et al., 2001). Nowadays, increasing evidence suggests that degenerative changes in cholinergic systems are due to age-related oxidative stress (Reddy, 2007), which means the free radicals also play an important role in the progression of AD. According to the free radical theory (Valko et al., 2007), the generation of reactive oxygen species (ROS) or free radicals can lead to cell and tissue damage, resulting in aging and untimely cell apoptosis, which are both involved in the pathogenesis of AD. The ROS can be scavenged by endogenous antioxidants including superoxide dismutases (SOD) and glutathione peroxidase (GSH-Px). Malondialdehyde (MDA) is a by-product of lipid peroxidation induced by free radicals and is widely used as a biomarker of oxidative stress (Aydin et al., 2007). One approach against ROS in neurodegenerative disorders is to enhance oxidative defenses via antioxidants (Valko et al., 2007).

Huperzine A (HA), a novel alkaloid isolated from the Chinese herb *Huperzia serrata*, is a highly selective, potent and reversible acetylcholinesterase (AChE) inhibitor (Wang et al., 2006), which has been widely used for AD therapy in China. Compared with the other AChE inhibitors, such as tacrine, donepezil and rivastigmine mentioned above, HA has a higher oral bioavailability, longer duration of AChE inhibitory action and better penetration through the blood-brain barrier (Bai et al., 2000). Beyond the potent AChE inhibition, HA

* Corresponding authors. G. Luo is to be contacted at Department of Chemistry, Tsinghua University, Beijing 100084, PR China. Tel./fax: +86 10 62781688. Q. Liu, School of Medicine, Tsinghua University, Beijing 100084, PR China. Tel./fax: +86 10 62797450.
E-mail addresses: luoga.mail@gmail.com (G. Luo), liuqf@mail.tsinghua.edu.cn (Q. Liu).

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possesses the ability to interfere with Aβ precursor protein metabolism, attenuate oxidative stress, protect neurons against apoptosis, and regulate the expression and secretion of nerve growth factor, holding great promise as a disease-modifying agent in the treatment of AD (Zhang et al., 2008; Zhang and Tang, 2006).

Ligustrazine, one of the active components of traditional Chinese medicine, Haoben Chuanxiong (*Ligusticum chuanxiong* Hort), is widely used in the treatment of cerebral vascular and cardiovascular diseases in clinic as an antioxidant (Xu and Shi, 2003). Ligustrazine phosphate (LP), the synthetic product of ligustrazine, has been demonstrated to play a protective role in neuroprotection, the possible mechanisms of which include inhibitory effects on the calcium overload, anti-inflammatory potential and anti-apoptotic activity (Cheng et al., 2007; Fan et al., 2006). Furthermore, recent studies have proved that ligustrazine (100 mg/kg, i.g.) can significantly improve the hippocampal cholinergic system function, attenuate oxidative damage, and therefore remarkably enhance the learning and memory abilities in D-galactose induced AD mice model (Zhao et al., 2008), suggesting that LP is a novel drug candidate for the treatment of AD.

As is well known, scopolamine, a muscarinic cholinergic receptor antagonist, has been widely adopted to study cognitive deficits in experimental animals. After i.p. injection of scopolamine, the cholinergic neurotransmission was blockaded, leading to cholinergic dysfunction and impaired cognition in rats (Oh et al., 2009). Recently, it has been reported that memory impairment induced by scopolamine in rats is associated with altered brain oxidative stress status (Fan et al., 2005). Therefore, rats with scopolamine-induced memory deficits were used as an animal model for screening anti-dementia drugs (Chen et al., 2008).

Since the etiology of AD involves multiple mechanisms, combined usage of two or more drugs aiming at diverse targets of the disease has attracted intense research, which is expected to act better than the single-target-aiming therapeutic strategy in the fight against AD (Youdim and Buccafusco, 2005). Recently, several combination therapies for AD have been evaluated in preclinical models to demonstrate that successful synergy of compounds can be achieved. Such combination therapies have been conducted for donepezil (an AChE inhibitor) and cilostazol (a cyclic AMP enhancer with anti-apoptotic and anti-inflammatory action) (Lee et al., 2007), donepezil and prucalopride (a 5-HT4 receptor antagonist) (Cachard-Chastel et al., 2008), and, most recently, rivastigmine (an AChE inhibitor) and methylthioninium chloride (an antioxidant) (Deiana et al., 2009). Principle outcomes met with enhanced success relative to the respective mono-therapy. Based on HA and LP having different mechanisms of action at the cellular level, coadministration of HA and LP might prove beneficial for the treatment of AD, however, there's no related literature up till now. Therefore, in this study, we were interested in investigating whether coadministration of HA and LP could effectively reverse the cognitive symptoms in the scopolamine-induced amnesia rats by employing the Morris water maze test. Furthermore, the activities of AChE and antioxidant enzymes, such as SOD and GSH-Px, and the levels of MDA within the cerebral cortex and hippocampus of the rats were respectively evaluated to illuminate the biochemical mechanism of the anti-amnesia effect.

2. Materials and methods

2.1. Drugs and reagents

Huperzine A (purity>97.0%) was supplied by Wanbang Pharmaceutical Company (Zhejiang, China). Ligustrazine phosphate (purity>98.0%) was purchased from Yanjing Pharmaceutical Company (Beijing, China). Scopolamine hydrochloride was obtained from Sigma-Aldrich (St Louis, MO, USA) and dissolved in 0.9% saline at the concentration of 0.6 mg/mL. Commercial kits used for determination of AChE, SOD, GSH-Px and MDA were purchased from Jiancheng Institute of Biotechnology (Nanjing, China).

2.2. Animals and drug administration

Male Sprague–Dawley rats, weighing 230–270 g, were supplied by the Experimental Animal Division of Peking University Health Sciences Center (Beijing, China). All rats were housed under standard condition of temperature 25 °C, 12 h/12 h light dark cycles and fed with a standard laboratory diet and water ad libitum. All animal studies were approved by the Institutional Animal Care and Use Committee of Tsinghua University (Beijing, China).

The rats were randomly divided into five groups (10 rats in each group): control group, scopolamine group (Sco group), HA group, LP group, and coadministration group (Coa group). Rats in the HA and LP groups were intragastrically (i.g.) administered HA (0.14 mg/kg) and LP (110 mg/kg), respectively, and rats in the Coa group were i.g. administered HA (0.14 mg/kg) and LP (110 mg/kg) simultaneously for 10 consecutive days. Rats in the control and Sco groups were treated similarly with corresponding volumes of saline. All rats except the control group received scopolamine hydrochloride (1.5 mg/kg body weight, i.p.) 30 min prior to the Morris water maze test.

The chosen doses for HA and LP were based on the administration dosages for human being. The administration dosage for HA tablet on the market in treatment of AD is 0.4 mg per day and the administration dosage for LP tablet in treatment of cardiovascular diseases is 300 mg per day. However, there's few literature referred to the oral dose for LP in the treatment of AD. On the basis of these information, we calculated the doses for HA and LP according to the dose conversion among different animals and human beings (Huang et al., 2004), and the chosen doses were close to those reported in the literatures (Fan et al., 2005; Oh et al., 2009).

2.3. Morris water maze test

2.3.1. Equipment and condition

The Morris water maze test, which is a widely accepted method for memory testing, was performed following the method described in previous reports (Sethi et al., 2009). The experimental apparatus consisted of a black circular water pool (120 cm diameter × 45 cm height), containing water (20 ± 1 °C) to a depth of 30 cm, and surrounded by extra maze distal visual cues of different shapes, sizes and colors. The pool was divided into four equal quadrants. A black circular hidden platform, 15 cm diameter, was centered constantly in the middle of the southwest quadrant, and was placed 1 cm below the water surface so that the rat could escape from swimming. The swimming pool was monitored by a video camera connected to a video recorder and a computer, which could recognize the rat and record its escape latency and swimming paths automatically when the rat was putted into the pool.

2.3.2. Morris water maze procedure

On the first day (day 0) of the experimental procedure, rats performed a single trial of 120 s, without the platform present, to get used to the pool. In the acquisition training, rats performed four training trials per day for four consecutive days (days 1, 2, 3 and 4) with the escape platform hidden in the middle of the southwest quadrant. The starting position was changed randomly for each trial and each rat was allowed to swim freely until it found the submerged platform or until 120 s elapsed. If the rat found the platform, it was allowed to remain there for 15 s and then returned to its home cage. If it was unable to find the platform within 120 s, it was then placed on the platform for 15 s and a maximum score of 120 s was assigned. In each training trial, the latency to escape onto the hidden platform was measured. The results on swimming distance were not shown as, in all cases, they were parallel with latencies.

On the day after the acquisition training (day 5), the probe trail test was carried out by removing the platform and allowing each rat to swim freely for 120 s inside the pool. For each rat, the time spent and the distance swam in the target quadrant in which the platform had been placed during the acquisition training were recorded.

2.4. Preparation of tissue samples

After the Morris maze water test, rats were sacrificed by cervical dislocation 40 min after injection of scopolamine, and the brain was immediately removed. The cerebral cortex and hippocampus were dissected and stored at −80 °C until the biochemical studies. Before detection, each part of the brain tissue was rapidly homogenized in ice-cold saline at 13 000 rpm in a Potter homogenizer, respectively. The homogenates were centrifuged at 3500 rpm at 4 °C for 15 min, and the supernatant was collected for assay. Protein concentrations were determined by the BCA assay (Cowin Biotech, Beijing, China).

2.5. Measurements of AChE, SOD, GSH-Px activities and MDA level in rats' cerebral cortex and hippocampus

The activities of AChE calculated as units per microgram of protein were measured in homogenates from the cerebral cortex and hippocampus, respectively, according to the colorimetric method (Ellman et al., 1961).

The activities of SOD were assayed based on the mechanism that the oxidation of oxymine by the xanthine–xanthine oxidase system (Ōyanagui, 1984) could be inhibited by SOD, and the hydroxylamine nitrite produced by the oxidation of oxymine had an absorbance peak at 550 nm. One unit (U) of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%. The SOD activity was expressed as units per microgram of protein.

The activities of GSH-Px were determined by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H_2O_2 catalyzed by GSH-Px, and one unit (U) of GSH-Px was defined as the amount that reduced the level of GSH by 1 μmol L^{-1} in 1 min per milligram protein.

The levels of MDA were determined by thiobarbituric acid reaction (TBAR) method, which can be measured at the wavelength of 532 nm by reacting with thiobarbituric acid (TBA) to form a stable chromophoric production (Ohkawa et al., 1979). The data was expressed as nanomoles per milligram of protein.

2.6. Statistical analysis

The statistical analysis was performed using OriginPro 7.5 software. Each data value is presented as the mean ± S.D. The data in the Morris water maze test and in the biochemical studies were analyzed by One-way ANOVA followed by a post hoc Bonferroni's multiple comparison test. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

3. Results

3.1. Morris water maze test

The Morris water maze test is a sensitive method for revealing the impairment of spatial learning and memory. The escape latency of finding a platform in the water maze was used as a parameter for evaluating performance in tested rats. For each rat, the escape latency was averaged for four trials, and the results are shown in Fig. 1. In the acquisition training, rats were trained for four consecutive days to

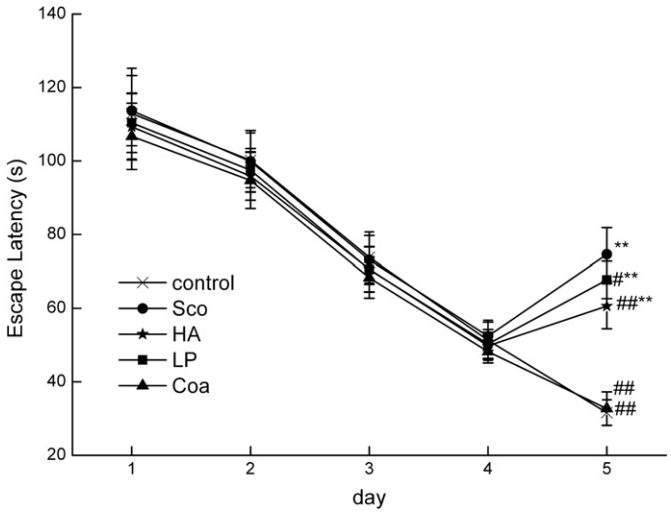

Fig. 1. Effects of the treatment with HA alone, LP alone and Coa on escape latency of the scopolamine-induced amnesia rats in the Morris water maze test. Each data point represents the mean (±S.D.) escape latency of four trails for ten rats performed each day. **Results significantly differ from the values of the control group: P<0.01. #Results significantly differ from the values of the Sco group: P<0.05. ##Results significantly differ from the values of the Sco group: P<0.01.

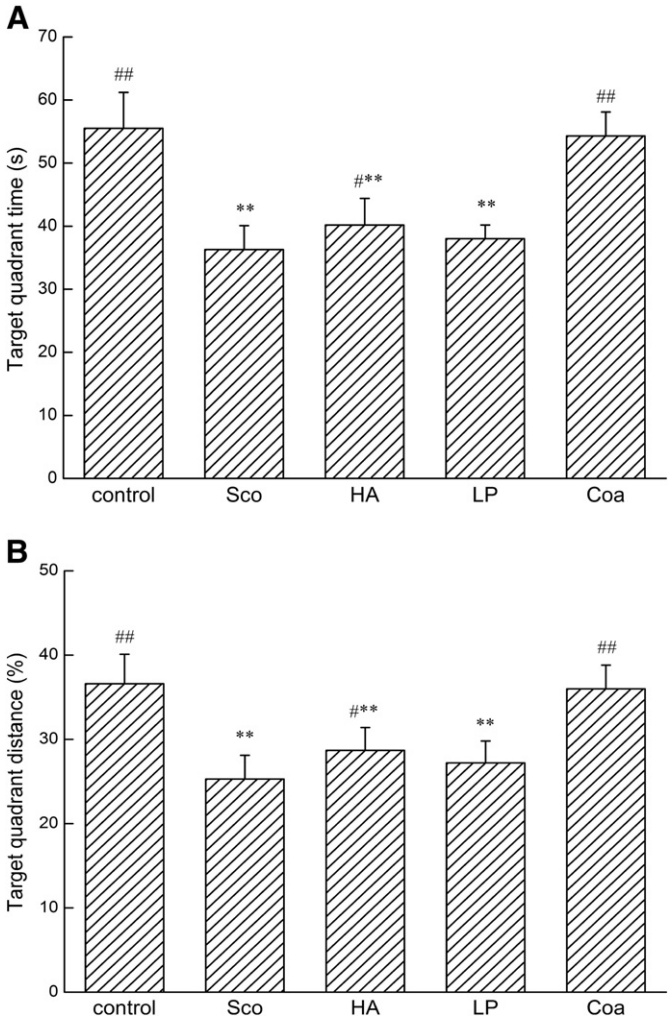

Fig. 2. Effects of the treatment with HA alone, LP alone and Coa on probe trial test of the scopolamine-induced amnesia rats in the Morris water maze test. The values are expressed as mean ± S.D. (n = 10). (A) The time of swimming for each rat spent in the target quadrant within 120 s. (B) The percent of the distance for each rat spent in the target quadrant within 120 s. **Results significantly differ from the values of the control group: P<0.01. #Results significantly differ from the values of the Sco group: P<0.05. ##Results significantly differ from the values of the Sco group: P<0.01.

learn where the hidden platform was located. All the rats significantly shortened the escape latency during the acquisition training, and there was no significant difference in the escape latencies among the groups on the same day.

On the fifth day, the rats received a scopolamine injection before the probe trail test in order to induce memory deficits (Fig. 1). Significant differences among the groups were obtained [$F(4, 45) = 134.38$, $P<0.001$]. Whereas rats in the control group preferentially spent their time swimming in the pool quadrant where the platform had been located during the acquisition phase, rats in the Sco group did not show such preference. Treatment with Coa totally reversed the memory deficits induced by scopolamine (Fig. 1) with the escape latency decreasing from 106.70 ± 9.03 s on the first day to 32.70 ± 4.55 s on the fifth day. Treatment with HA has shown a significant preventive effect against scopolamine-induced memory impairment. The escape latency decreased from 109.30 ± 9.03 s on the first day to 60.60 ± 6.17 s on the fifth day, and a similar effect was also observed in the LP group, with the escape latency decreasing from 110.40 ± 8.10 s the first day to 67.70 ± 5.08 s the fifth day, which indicated that the memory deficits induced by scopolamine were only partially reversed in the HA group or the LP group.

In the probe trial test, the average time and the percentage of distance for each rat spent in target quadrant were calculated to make the comparison between groups, and the results are shown in Fig. 2A and B. Compared with the Sco group, rats in the Coa group [$F(1, 18) = 72.91$, $P<0.01$; $F(1, 18) = 110.88$, $P<0.01$] or in the HA group [$F(1, 18) = 7.36$, $P<0.05$; $F(1, 18) = 4.64$, $P<0.05$] significantly shown more time and distance in the target quadrant, respectively. Although a similar effect was observed in the LP group, there was no statistically significant difference occurred between the Sco group and the LP group. Only the rats in the Coa group were powerful enough to induce the same preference in the target quadrant as the rats in the control group.

3.2. Biochemical studies

3.2.1. Effects of the coadministration of HA and LP on AChE activities in the cerebral cortex and hippocampus of rats

Since acetylcholinesterase inhibitors are known to antagonize scopolamine-induced amnesia (Chen et al., 2008; Lima et al., 2009), the effects of treatment with Coa on AChE activities in the cerebral cortex and hippocampus of rats were evaluated, respectively, and the results are shown in Table 1. The AChE activities in the cerebral cortex and hippocampus of rats treated with scopolamine were significantly increased in comparison with the control group, respectively [$F(1, 18) = 39.64$, $P<0.01$; $F(1, 18) = 446.18$, $P<0.01$]. Compared with the Sco group, the AChE activities within the cerebral cortex and hippocampus in the Coa group were significantly inhibited by 27% and 42%, respectively, and the AChE activities in the HA group were significantly inhibited by 22% and 39%, respectively. The AChE activities in the LP group were also suppressed compared to the Sco group, however, no statistically significant difference was observed between them. Treatment with Coa could reduce the activities of AChE within both of the cerebral cortex and hippocampus to the similar level of the rats in the control group. However, only the activity of AChE within the hippocampus of rats in the HA group reversed to the similar level of the rats in the control group, but not within the cerebral cortex. Compared to the rats in the control group, rats in the LP group have shown much higher activities of AChE within the cerebral cortex [$F(1, 18) = 80.71$, $P<0.01$] and hippocampus [$F(1, 18) = 240.34$, $P<0.01$], respectively.

Table 1
The effect of the treatment with HA alone, LP alone and Coa on AChE activities within the cerebral cortex and hippocampus of the scopolamine-induced amnesia rats.

Groups	AChE (U/mg protein)	
	Cerebral cortex	Hippocampus
Control	$0.89 \pm 0.06^{\#\#}$	$0.69 \pm 0.05^{\#\#}$
Sco	$1.24 \pm 0.06^{**}$	$1.20 \pm 0.05^{**}$
Coa	$0.90 \pm 0.08^{\#\#}$	$0.70 \pm 0.03^{\#\#}$
HA	$0.97 \pm 0.05^{\#\#*}$	$0.73 \pm 0.07^{\#\#}$
LP	$1.13 \pm 0.04^{**}$	$1.16 \pm 0.08^{**}$

The values are expressed as mean ± S.D. (n = 10). *Results significantly differ from the values of the control group: $P<0.05$. **Results significantly differ from the values of the control group: $P<0.01$. ##Results significantly differ from the values of the Sco group: $P<0.01$.

Table 2
The effect of treatment with HA alone, LP alone and Coa on SOD activity, GSH-Px activity and MDA level within the cerebral cortex of the scopolamine-induced amnesia rats.

Groups	SOD (U/mg protein)	GSH-Px (U/mg protein)	MDA (nmol/mg protein)
	Cortex	Cortex	Cortex
Control	$98.62 \pm 1.39^{\#\#}$	$116.75 \pm 9.68^{\#\#}$	$4.04 \pm 0.12^{\#\#}$
Sco	$51.07 \pm 1.21^{**}$	$84.58 \pm 5.28^{**}$	$8.50 \pm 0.31^{**}$
Coa	$97.33 \pm 1.31^{\#\#}$	$112.10 \pm 9.10^{\#\#}$	$4.07 \pm 0.18^{\#\#}$
HA	$90.11 \pm 1.73^{\#\#**}$	$99.37 \pm 4.49^{\#\#**}$	$5.62 \pm 0.15^{\#\#**}$
LP	$96.12 \pm 2.25^{\#\#}$	$110.42 \pm 4.26^{\#\#}$	$4.08 \pm 0.18^{\#\#}$

The values are expressed as mean ± S.D. (n = 10). **Results significantly differ from the values of the control group: $P<0.01$. ##Results significantly differ from the values of the Sco group: $P<0.01$.

3.2.2. Effects of the coadministration of HA and LP on antioxidant enzyme activities and MDA levels in the cerebral cortex and hippocampus of rats

To further elucidate the biochemical mechanism of the anti-amnesic activity of drug coadministration in brain tissues, the effects on the activities of antioxidant enzymes and MDA levels were determined, and the results are shown in Tables 2 and 3. Compared with the control group, the administration of scopolamine resulted in a significant reduction of GSH-Px [$F(1, 18) = 85.11$, $P<0.01$; $F(1, 18) = 310.34$, $P<0.01$] and SOD activities [$F(1, 18) = 1159.42$, $P<0.01$; $F(1, 18) = 546.21$, $P<0.01$] in the cerebral cortex and hippocampus, respectively, and the MDA levels were significantly increased [$F(1, 18) = 1738.40$, $P<0.01$; $F(1, 18) = 138.38$, $P<0.01$] in both of the brain tissues. Treatment with Coa or LP alone significantly recovered the activities of GSH-Px and SOD in the cerebral cortex and hippocampus of the scopolamine-induced amnesic rats to the similar level of the rats in the control group, and the increase in MDA levels induced by scopolamine was also recovered to the similar level of the rats in the control group. An analogical amelioration was also observed in the HA group, however, neither the activities of the antioxidant enzymes nor the levels of MDA were recovered to the similar level of the rats in the control group.

4. Discussion and conclusion

During the acquisition training in the Morris water maze test, neither HA nor LP could change the behavioral performance of the

Table 3
The effect of treatment with HA alone, LP alone and Coa on SOD activity, GSH-Px activity and MDA level within the hippocampus of the scopolamine-induced amnesia rats.

Groups	SOD (U/mg protein)	GSH-Px (U/mg protein)	MDA (nmol/mg protein)
	Hippocampus	Hippocampus	Hippocampus
Control	$62.64 \pm 2.19^{\#\#}$	$31.96 \pm 1.73^{\#\#}$	$2.37 \pm 0.13^{\#\#}$
Sco	$42.30 \pm 1.66^{**}$	$21.02 \pm 0.93^{**}$	$4.50 \pm 0.15^{**}$
Coa	$61.37 \pm 2.28^{\#\#}$	$31.26 \pm 1.27^{\#\#}$	$2.39 \pm 0.09^{\#\#}$
HA	$53.25 \pm 1.81^{\#\#**}$	$24.33 \pm 1.18^{\#\#**}$	$3.56 \pm 0.11^{\#\#**}$
LP	$60.70 \pm 1.02^{\#\#}$	$30.79 \pm 0.83^{\#\#}$	$2.51 \pm 0.18^{\#\#}$

The values are expressed as mean ± S.D. (n = 10). **Results significantly differ from the values of the control group: $P<0.01$. ##Results significantly differ from the values of the Sco group: $P<0.01$.

normal rats since there was no significant difference in the escape latencies among the groups on the same day, which indicated that both of HA and LP had no effect on the normal rats. In this study, rats with scopolamine-induced amnesia were used as an animal model for investigating whether the coadministration of HA and LP could effectively reverse scopolamine-induced memory deficits in rats. After the rats were treated with scopolamine, performance on several reference memory tasks was disrupted, such as discriminating object and delaying matching to target position in the Morris water maze test. Furthermore, the activities of AChE increased, the activities of SOD and GSH-Px decreased, and the levels of MDA increased significantly within the cerebral cortex and hippocampus, indicating that scopolamine could not only interrupt the cholinergic neurotransmission but also trigger the oxidative stress in the brain of the rats, which were both involved in the pathogenesis of AD. The results were in line with the previous literatures (Chen et al., 2008; Fan et al., 2005).

On the fifth day before the probe trail test, the memory deficits induced by scopolamine were totally recovered to the similar status of the normal rats when the rats were treated with Coa, in contrast, the memory deficits were only partially reversed when the rats were treated with HA or LP alone, which suggested that treatment with Coa could attenuate the learning and memory impairment induced by scopolamine. Moreover, the results in the probe trial test further signified the better effect of treatment with Coa on the behavioral performance of scopolamine-induced amnesia rats compared with that of treatment with individual drug.

Cholinergic dysfunction and oxidative stress are two pathogeneses of AD, and degenerative changes in cholinergic systems are related to oxidative stress as mentioned above. Moreover, AChE activity, SOD and GSH-Px activities and MDA level are main biomarkers of the cholinergic function and oxidative status, respectively. Therefore, the effects on these activities/levels of the brain tissues were evaluated to elucidate the biochemical mechanism of the anti-amnesic behavioral performance. The results in biochemical studies have shown that HA could not only inhibit the activity of AChE but also possessed a few antioxidant activities within the cerebral cortex and hippocampus, which was according to the previous researches (Zhang and Tang, 2006). In addition, LP as a scavenger of free radicals also had a few effect on AChE inhibition. Based on the varied mechanisms of both drugs targeting on different pathogeneses of AD, treatment with Coa could significantly inhibit AChE activities, increase SOD and GSH-Px activities and also reduce MDA levels within the cerebral cortex and hippocampus in the scopolamine-induced memory deficit rats, and most importantly, reverse these activities/levels to the similar status of the normal rats, which surpassed those of treatment with either drug. Altogether, treatment with Coa could exert improved effects on these biomarkers, ameliorating cholinergic dysfunction and oxidative stress simultaneously, which was also indirectly reflected by the improvement in the behavioral performance.

In summary, the improvement of the efficacy by this polytherapy might not only contribute to behavioral improvement, but also slow down the progression of AD by protecting the cholinergic system and mitigating oxidative stress. Hence, coadministration of HA and LP could be considered as a strategy to prevent or slow down the development of AD.

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