The mixture of procyanidins extracted from the lotus seed pod and bilobalide ameliorates scopolamine-induced memory impairment in mice

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Abstract: Objective To study the co-effect of procyanidins extracted from the lotus seed pod (LSPC) and bilobalide (BIL) on ameliorating scopolamine-induced learning and memory impairment in young mice. **Methods** Fifty male Kunming mice with similar learning and memory capabilities were selected by Morris water maze test and were randomized into 5 groups ($n=10$ in each group): control group, scopolamine group, L-(LSPC+BIL) group (50 mg/kg LSPC+10 mg/kg BIL), M-(LSPC+BIL) group (100 mg/kg LSPC+20 mg/kg BIL), H-(LSPC+BIL) group (150 mg/kg LSPC+30 mg/kg BIL). Scopolamine model with impaired learning and memory was established by scopolamine treatment (1 mg/kg), and after 10 min mice were tested. In L-, M-, and H-(LSPC+BIL) groups, mice were treated with LSPC and BIL ig. for 30 days, while mice in the other 2 groups were treated with normal saline ig. instead. After the 30-day's treatment, the co-effect of LSPC and BIL on learning and memory was tested by Morris water maze and the step-down avoidance tests. **Results** The memory impairment caused by scopolamine in young mice could be ameliorated by co-treatment of LSPC and BIL, as indicated by significantly shorter escape latency and swimming distance in the Morris water maze test, when compared with those in the scopolamine group. In the step-down avoidance test, mice in all the 3 dose groups showed significantly smaller number of errors and longer latency than mice in the scopolamine group did. **Conclusion** Co-treatment of LSPC and BIL can ameliorate scopolamine-induced learning and memory impairment in young mice.

Keywords: lotus seed pod procyanidins; bilobalide; scopolamine; mice; learning and memory

1 Introduction

With the improvement of human society and rapid economic development, aging is becoming a worldwide issue and the cognitive performance in the elderly declines with

aging. The mechanisms of senile dementia are complex and still remain to be fully elucidated. However, increasing evidences demonstrate that cholinergic deficit is closely correlated with the severity of cognition dysfunction and memory loss in senile dementia patients as well as in aged human^[1].

At present, although acetylcholine replacement therapy has been the first-line clinical therapy for senile dementia and does exhibit some beneficial effects, it provides symptomatic relief only. Moreover, the therapy often induces side effects in patients, such as salivation, lacrimation, decreased heart rate, and CNS activation^[2]. Clinically, it is imperative to

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find other effective treatments for senile dementia, with little or even no damage to human body.

Being a common compound in plants, fruits, vegetables and seeds, procyanidins has been reported to exert strong biological effects on cardiovascular disease, cancer and certain neurodegenerative disorders^[3]. Lotus seeds contain abundant procyanidins and exist as both food and medicine. Some studies have suggested that procyanidins may play a potential role in enhancing cognition^[4, 5], which gives us a hint that it may also lead to some beneficial improvements in the central cholinergic system.

Bilobalide is the extract from ginkgo biloba leaves, a herbal very popular in Chinese herbal medicine and has been used to treat a variety of disorders in thinking, learning and memory, based on its ability to improve blood flow in brain and its antioxidant properties^[6]. Recent evidence suggests that bilobalide may also have direct effects on the cholinergic system, which might explain its enhancing effects in both acute and chronic cognitions^[6].

Presently, the co-effect of procyanidins extracted from the lotus seed pod (LSPC) and bilobalide (BIL) in enhancing cognitive performance remains unknown. The present study aims to assess the co-effects of LSPC and BIL in ameliorating scopolamine-induced memory impairment in mice by Morris water maze test and step-down avoidance test.

2 Materials and methods

2.1 Materials (-)-scopolamine hydrobromide, LSPC and BIL were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals and organic solvents were all of analytical grade.

2.2 Animals A total of 70 male Kunming mice (2-month-old, 18–22 g, provided by the Experimental Animal Center of Tongji Medical College, Huazhong Science and Technology University) were used in this experiment. Each 5 mice were kept in one cage. Mice were housed under standard temperature ($24 \pm 1^\circ\text{C}$) and diurnal conditions (light–dark: 08:00–20:00), with free access to laboratory chow and tap water *ad libitum*. The animals were cared for in accordance with the Guiding Principles in the Care and Use of Animals. The experiment was approved by Tongji Medical College Council

on Animal Care Committee, Huazhong University of Science and Technology, China.

2.3 Model establishment and co-treatment of LSPC and BIL

Fifty male Kunming mice with similar learning and memory capabilities were selected through Morris water maze test and were randomized into 5 groups ($n=10$ in each group): control group, the scopolamine group, L-(LSPC+BIL) group (50 mg/kg LSPC+10 mg/kg BIL), M-(LSPC+BIL) group (100 mg/kg LSPC+20 mg/kg BIL), H-(LSPC+BIL) group (150 mg/kg LSPC+30 mg/kg BIL). LSPC and BIL solutions were freshly daily prepared throughout the experimental period. Mice in L-, M-, and H-(LSPC+BIL) groups were given a daily treatment of 50, 100, 150 mg/kg LSPC and 10, 20, 30 mg/kg BIL *ig.*, respectively, for 30 days. Mice in the control group and the scopolamine group were given an equivalent amount of normal sodium, with the same *ig.* procedure.

On each day of the behavioral test period, mice in L-, M-, and H-(LSPC+BIL) groups were given the mixture of LSPC and BIL 1 h before the first trial session. Animals in L-, M-, and H-(LSPC+BIL) groups and the scopolamine group were treated with scopolamine (1 mg/kg, *i.p.*) dissolved in normal saline 10 min before the first trial session during the training phase of both behavioral tests. Mice in the control group received vehicle only.

2.4 Behavioral tests

2.4.1 Morris water maze test^[7] The Morris water maze was conducted in a large circular pool with a diameter of 120 cm and 4 side walls approximately 45 cm high, containing no internal cues, stimuli, markings or objects, but surrounded by stable, salient extra-maze cues. The pool was filled with water at a temperature of $22 \pm 1^\circ\text{C}$ to a depth of about 30 cm. The pool was conceptually divided into 4 quadrants with equal areas (NE, SE, SW and NW). A circular escape platform (10 cm in diameter and 29 cm in height) was placed into the tank at a fixed position in the center of the SW quadrant, and was 40 cm away from the wall. Black nontoxic carbon ink was added to the pool to make the water opaque. The top surface of the platform was approximately 1 cm below the water surface, with a rough surface to make it easy for the mice to climb on.

Morris water test was conducted for 5 days and all ex-

periments were carried out between 10:00 and 15:00. Each mouse underwent 3 trials per day with an inter-trial interval for 3 min. During the test trial, the mouse was released into the water at one of the 3 different starting positions (in 3 different quadrants that did not contain the platform), with its head facing the wall. And then the mouse was allowed to swim for 90 s to search for the hidden platform. If it failed to locate the platform within 90 s, escape would be assisted and escape latency was recorded as 90 s. At the end of each trial, each mouse would stay on the platform for 10 s. The sequence of the starting positions remained the same for all the mice within one session, but changed on each day.

During each trial session, escape latency (the time taken to find the platform) in s, swimming distance (length of swim path to reach the platform) in cm, and average swimming speed to reach the platform in cm/s were recorded using an overhead video camera-based Ethovision System (Nodulus, Wageningen, The Netherlands).

2.4.2 Step-down avoidance test The step-down avoidance test was performed using a shock grid apparatus (STT-2, Institute of Materia Medica, Chinese Academy of Medical Sciences) which consisted of a round rubber platform (4.5 cm in diameter and 4.5 cm in height) fixed in the left end of an acrylic box ($33 \times 11 \times 11 \text{ cm}^3$) and an electrifiable metallic grid floor connected with the electric shock generator. Initially, mouse was placed on the safe platform. When it stepped down with all the 4 paws onto the floor, it would receive a foot shock (1 s, 36 V) and it was normal reaction to escape and be back onto the platform.

The test was performed 7 days after the Morris water maze as previously described^[8], and the experiments were conducted between 14:00 and 16:00. Prior to the test, the mouse was allowed an adaptation period of 3 min in the box without electric shock, then it was placed on the platform. The training trial began once the animal stepped down from the platform with its 4 paws touching the grid floor. The mouse would escape and be back up onto the platform on receiving the electric shock. The number of errors (the mouse steps down from the platform and gets a shock) within 5 min was recorded. After an interval of 24 h (testing trial), the mouse was placed on the safe board and steel rods that were

disconnected with the electric shock generator. Step-down latency (duration of staying on the platform) in s was recorded to assess the level of retention of passive avoidance. If the mouse did not step down to the grid floor within 5 min, a ceiling score of 5 min was assigned.

2.5 Statistical analysis Data were presented as mean $\pm$ SEM. The parameters obtained from the Morris water maze were analyzed using repeated measures and multivariate analysis of variance (ANOVA) process with factors of group and day as the between subject factor and the within subject factor, respectively [group (5) $\times$ day (5)], followed by the least significant difference (Fisher PLSD) post hoc test in case the overall differences were significant ($P < 0.05$)^[9]. Data from the step-down avoidance test were analyzed by nonparametric statistics (Kruskal-Wallis analysis of variance followed by Mann-Whitney U test, two-tailed). All the analysis was performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). $P < 0.05$ was considered as statistically significant.

3 Results

3.1 The co-effect of LSPC and BIL revealed by Morris water maze test The co-effect of LSPC and BIL on spatial learning was evaluated by Morris water maze test. There were significant main effects in factors of day ($P < 0.001$) and group ($P < 0.001$).

As shown in Table 1, there was no significant difference in the escape latency from day 1-4 in the scopolamine group ($P > 0.05$), however, the escape latency on day 5 was significantly shorter than that on day 1 ($P < 0.05$). Moreover, scopolamine-treated mice exhibited longer escape latency than the control mice did during all sessions ($P < 0.05$). On day 5, mice in L-, M-, and H-(LSPC+BIL) groups all exhibited significantly shorter escape latency than mice in the scopolamine group did ($P < 0.05$).

As shown in Table 2, swimming distance of the scopolamine group was significantly longer than that of the control group ($P < 0.05$) during the training days except on day 2. Swimming distance of M-(LSPC+BIL) group on day 4 was significantly shorter than that of the scopolamine group; while on day 5, the swimming distances of L-, M-, and H-

Table 1. The co-effect of LSPC and BIL on the escape latency in the Morris water maze test(mean±SEM)

Groups	1d	2d	3d	4d	5d
Control	56.513±5.963*	52.273±7.398*	38.567±7.418*	36.307±7.418*	33.953±4.579*
Scopolamine	84.86±2.015	80.133±2.897	78.067±2.810	75.86±5.115	69.12±4.428 [#]
L-(LSPC+BIL)	81.367±3.334	0.887±6.907	63.853±8.525	58.887±6.294	44.3±8.303*
M-(LSPC+BIL)	74.115±4.447	60.02±7.260	58.813±7.689	53.207±7.689	45.473±4.275*
H-(LSPC+BIL)	71.227±5.589	63.42±5.508	61.927±6.071	53.207±5.042	39.260±8.394*

* $P < 0.05$, vs the scopolamine group; [#] $P < 0.05$, vs 1d in the scopolamine group.

Table 2. The co-effect of LSPC and BIL on the swimming distance in the Morris water maze test(mean±SEM)

Groups	1d	2d	3d	4d	5d
Control	1000.272±118.037*	923.725±124.438	663.198±87.239*	630.994±104.260*	611.376±94.460*
Scopolamine	1416.193±89.423	1270.56±118.034	1337.461±143.149	1471.581±159.359	1404.97±126.964
L-(LSPC+BIL)	1169.894±99.331	1308.313±125.109	1143.293±149.762	1110.27±138.151	843.627±163.344 [#]
M-(LSPC+BIL)	1213.186±76.708	1180.619±120.689	1151.991±110.237	1034.176±115.683 [#]	1001.214±98.883 [#]
H-(LSPC+BIL)	1190.842±134.794	1165.86±112.253	1162.027±98.883	1014.401±136.308	854.324±161.428 [#]

* $P < 0.01$, vs the scopolamine group; [#] $P < 0.05$, vs the scopolamine group.

Table 3. The co-effect of LSPC and BIL on latency and number of errors in the step-down avoidance test(mean±SEM)

Groups	Latency(s)	Number of errors
Control	178.3000±29.1338*	1.9000±0.3786*
Scopolamine	18.6000±8.5456	8.6000±1.1274
L-(LSPC+BIL)	59.5000±18.6954*	5.0000±1.1643 [#]
M-(LSPC+BIL)	94.5000±25.2728*	3.8182±0.7841 [#]
H-(LSPC+BIL)	97.6000±24.8963*	4.4000±0.8458 [#]

* $P < 0.01$, vs the scopolamine group; [#] $P < 0.05$, vs the scopolamine group.

(LSPC+BIL) groups were all significantly shorter than that of the scopolamine group ($P < 0.05$).

Besides, there was no significant difference in the average speed of swimming among all the groups during all training days (data not shown).

3.2 Co-effect of LSPC and BIL on latency and number of errors in the step-down avoidance test As shown in Table 3, scopolamine treated mice exhibited increased number of errors ($P < 0.01$) and reduced latency ($P < 0.01$) than the control mice did in the step-down avoidance test. Moreover, mice in L-, M-, and H-(LSPC+BIL) groups all showed longer latency

and less number of errors than mice in the scopolamine group did ($P < 0.01$ and $P < 0.05$, respectively).

4 Discussion

The cholinergic hypothesis proposes that the dysfunction of the cholinergic system contributes to the memory decline in aging and dementia (Bartus *et al.*, 1982). Cholinergic transmission through muscarinic acetylcholine receptors (mAChR) has been implicated for its involvement in maintaining brain functions such as learning and memory. As an antagonist for muscarinic receptor, scopolamine can

cause impairment in learning and to some degree in memory processing^[10]. In our studies, mice receiving scopolamine treatment showed significant increases in escape latency and swimming distance in Morris water maze, and also shorter step-down latency and greater number of errors in step-down avoidance test. Based on these findings, the mouse model of scopolamine-induced memory impairment is considered to be successfully established and can be used to evaluate the co-effect of LSPC and BIL on memory improvement.

Procyanidins are major dietary polyphenols made of elementary flavan-3-ol (epi)catechin units. They can protect against oxidative stress through both free radical trapping and redox-active metals chelating^[11]. For example^[12], procyanidins can interact with the polar headgroup of lipids to increase the membrane fluidity, protecting against and repairing irradiation-induced DNA damage. As a free radical scavenger, it is not only more powerful than well-established antioxidants such as vitamins C and E, but also a highly-effective vitamin C potentiator. Recent research indicates that procyanidins is crucial in controlling synapse growth, in promoting an increase in dendritic spine density and in enhancing synaptic receptor density^[13]. In addition, extract from other herbs with cholinesterase properties, such as sage, has been proved to improve memory in the old. The safety evaluation and the effective doses of procyanidins have also been demonstrated^[14, 15].

Bilobalide's effect in enhancing cognition can be attributed to a combination of antagonistic effects of platelet activating factor, free radical scavenging activity and modulation of cholinergic function. Bilobalide's direct actions on cholinergic system include reducing scopolamine-induced amnesia, modulating pre-synaptic choline uptake and acetylcholine release, up-regulating expressions of post-synaptic muscarinic receptors. Bilobalide can also regulate cholinergic function indirectly by modulating the serotonergic system^[16].

In the Morris water maze test, the training trial was performed from day1-4, and spatial learning was evaluated on day 5. Compared to those in the scopolamine group, mice in L-, M-, and H-(LSPC+BIL) groups all showed shorter escape latency and swimming distances, which indicated the signifi-

cant enhancement in cognitive performance by LSPC and BIL. In the step-down avoidance test, mice in L-, M-, and H-(LSPC+BIL) groups exhibited longer step-down latency and less number of errors than mice in the scopolamine group did, which illustrated that scopolamine-induced memory deficit could be notably relieved by co-treatment of LSPC and BIL.

To sum up, the co-treatment of procyanidins extracted from the lotus seed pod and bilobalide could ameliorate scopolamine-induced memory impairment in mice. However, the mechanisms underlying these effects and the exact dose-dependent manner still remain unclear. Further studies are required for wide and effective clinical applications of the 2 compounds.

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References:

- [1] Terry AV, Buccafusco JJ. The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development. *J Pharmacol Exp Ther* 2003, 306(3): 821-827.
- [2] Masterman D. Cholinesterase inhibitors in the treatment of Alzheimer's disease and related dementias. *Clin Geriatr Med* 2004, 20(1): 59-68.
- [3] Koleckar V, Kubikova K, Rehakova Z, Kuca K, Jun D, Jahodar L *et al.* Condensed and hydrolysable tannins as antioxidants influencing the health. *Mini Rev Med Chem* 2008, 8(5): 436-447.
- [4] Devi A, Jolitha AB, Ishii N. Grape seed proanthocyanidin extract (GSPE) and antioxidant defense in the brain of adult rats. *Med Sci Monit* 2006, 12(4): BR124-129.
- [5] Gong Y, Liu L, Xie B, Liao Y, Yang E, Sun Z. Ameliorative effects of lotus seedpod proanthocyanidins on cognitive deficits and oxidative damage in senescence- accelerated mice. *Behav Brain Res* 2008, 194(1): 100-107.
- [6] Cong WH, Liu JX, Xu L. Effect of combination of extracts of ginseng and ginkgo biloba on neurotransmitters in whole brain of $\beta 2$ amyloid peptide 1240 treated rat. *Chin Pharmacia Bull* 2006, 22(6): 747-750.
- [7] Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* 1984, 11(1): 47-

- 60.
- [8] Rubio J, Dang HX, Gong MJ, Liu XM, Chen SL, Gonzales GF. Aqueous and hydro alcoholic extracts of Black Maca (*Lepidium meyenii*) improve scopolamine-induced memory impairment in mice. *Food Chem Toxicol* 2007, 45(10): 1882-1890.
- [9] Qiu H, Jin GQ, Jin RF, Zhao WK. Analysis of variance of repeated data measured by water maze with SPSS. *J Chin Integr Med* 2007, 5(1): 101-105.
- [10] Flood JF, Cherkin A. Scopolamine effects on memory retention in mice: a model of dementia? *Behav Neural Biol* 1986, 45(2): 169-184.
- [11] Gu LW, House SZ, Wu XL, Ou BX, Prior RL. Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *J Agric Food Chem* 2006, 54(11): 4057-4061.
- [12] Ronald L, Gu LW. Occurrence and biological significance of proanthocyanidin in the American diet. *Phytochemistry* 2005, 66(18): 2264-2280.
- [13] Jeremy PE. Food for thought: the role of dietary flavonoids in enhancing human memory, learning and neuro-cognitive performance. *Proc Nutr Soc* 2008, 67(2): 238-252.
- [14] Yamakoshi J, Saito M, Kataoka S, Kikuchi M. Safety evaluation of proanthocyanidin-rich extract from grape seeds. *Food Chem Toxicol* 2002, 40: 599-607.
- [15] Gong Y, Tang Y, Xiao J, Xie B. Effects of procyanidins extracted from lotus seedpod on learning and memory impairment in mice. *Acta Nutrim Sin* 2006, 28(4): 318-321.
- [16] Caltagirone A, Weiss G, Pantopoulos K. Modulation of cellular iron metabolism by hydrogen peroxide. Effects of H₂O₂ on the expression and function of iron responsive element-containing mRNAs in B6 fibroblasts. *J Bio Chem* 2001, 276 (23): 19738-19745.

莲房原花青素和银杏内酯联用对东莨菪碱所致小鼠记忆获得性障碍的改善作用

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摘要: **目的** 观察莲房原花青素和银杏内酯联用对东莨菪碱所致小鼠学习记忆获得性障碍的改善作用。**方法** 采用 Morris 水迷宫筛选出 50 只空间学习记忆能力相近的昆明种雄性小鼠, 随机分为 5 组, 即正常对照组、模型组、低剂量治疗组、中剂量治疗组、高剂量治疗组, 每组 10 只。行为学测试前 10 min 腹腔注射东莨菪碱, 建立小鼠记忆获得障碍模型。3 个治疗组分别灌胃 50 mg/kg 莲房原花青素+10 mg/kg 银杏内酯、100 mg/kg 莲房原花青素+20 mg/kg 银杏内酯、150 mg/kg 莲房原花青素+30 mg/kg 银杏内酯; 正常组和模型组以生理盐水代替。喂养 30 天后以 Morris 水迷宫法、跳台法行为学实验, 观察莲房原花青素和银杏内酯的联同作用。**结果** 在东莨菪碱所致小鼠记忆障碍模型中, 莲房原花青素和银杏内酯联用可缩短在 Morris 水迷宫中的潜伏期, 减少游泳距离。在跳台实验中莲房原花青素和银杏内酯联用可以延长平台停留期, 显著减少错误次数。**结论** 莲房原花青素和银杏内酯联用能够改善东莨菪碱所致小鼠学习记忆障碍。

关键词: 莲房原花青素; 银杏内酯; 东莨菪碱; 小鼠; 学习记忆