The Effects of Different Compatibilities of Qing'e Formula on Scopolamine-induced Learning and Memory Impairment in the Mouse

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Abstract

Background: The Qing'e formula (QEF) is a well-known traditional Chinese prescription that has been clinically employed for treatment of bone disease for hundreds of years. Objective: The present study aims to observe the effects of different compatibilities of QEF on the scopolamine-induced learning and memory impairment in the mouse, and further to explore its action mechanisms and compatibility rationality. Materials and Methods: The learning and memory alterations in the mouse were evaluated using the step-down test and Morris water maze (MWM) test; the acetylcholinesterase (AChE) activity and brain-derived neurotrophic factor (BDNF) expression in the hippocampus were measured using colorimetric method or immunohistochemistry. Results: The results showed that different compatibilities of QEF significantly prolonged latency in the step-down test, shortened escape latency in the navigation test, increased the percentage of residence time, and the percentage of swimming distance in the target quadrant in the probe trial session. In addition, our results also found that different compatibilities of QEF remarkably inhibited AChE activity and increased BDNF expression in the hippocampus of mice. What's more, the group after being treated with whole recipe (QF) showed the highest level of improvement. Conclusions: These findings not only suggest that QEF may effectively ameliorate cognitive deficits through inhibiting AChE activity and increasing BDNF expression in the hippocampus but also elucidate the rationality of QEF.

Keywords: Acetylcholinesterase, brain-derived factor, Learning and memory, Qing’e formula

INTRODUCTION

Alzheimer disease (AD) is a devastating neurodegenerative disorder affecting the millions of people. It reduces the ability of the individual to remain independent, brings a heavy burden on caregivers, and substantially increases health-care costs. The pathophysiology of AD is complex and unclear. There are many hypotheses to explain AD, such as cholinergic hypothesis, amyloid hypothesis, and tau hypothesis. Among them, the loss of cholinergic is more recognized, and its pathogenesis is widely studied at present. Therefore, current treatment prescribed in AD patients, for example, donepezil, is acetylcholinesterase inhibitors (AChEIs). However, these AChEIs have short half-lives and severe side effects, such as nausea, gastrointestinal upset, and diarrhea, which are the most frequent and important side effects of these therapies.

Traditional Chinese medicine (TCM) has been clinically used in China for thousands of years and has played an indispensable role in prevention and treatment of diseases, especially for complicated and chronic conditions. Now in TCM, several herbs and ingredients are combined according to strict rules to form prescriptions, which are referred to as formulas (方剂 in Chinese). In general, a classic formula is composed of four elements, i.e., an sovereign medicinal...
with one or more minister, assistant, and courier medicinals according to their different roles in the formula. Each formula comprises of one to several drugs. Moreover, the multiple components in TCM formulas exert a synergistic therapeutic effect, which is known as “formula compatibility.” Reasonable formula compatibility can achieve an overall effect of optimized combination. In recent years, TCM formulas have attracted a wide interest from Chinese and international researchers because of their few side effects, multicomponents, multipathways, and multigetargets.

Qing’e formula (QEF), originally recorded in “Taiping Huimin Heji Ju Fang (Prescriptions from the Great Peace Imperial Grace Pharmacy)” in the Song Dynasty (10th century CE), which is a classic traditional Chinese medicine recipe comprised of eucommnia cortex (EC), psoraleae fructus (PF), juglandis semen (JS), and garlic rhizoma (GR). It is officially recorded in Chinese pharmacopoeia for kidney-tonifying and bone-strengthening. QEF has been showed to have the efficacy of enhancing immune function, improving microcirculation, and being analgesic, antibacterial, and anti-inflammatory. It is famous in the clinical practice for its efficacy in the treatment of postmenopausal osteoporosis, coronary heart disease, acute exacerbation stage of chronic bronchitis, chloasma in recent years. Modern pharmacology research shown that EC had anti-amnesic activity through the inhibition of acetylcholinesterase (AChE) and protection of brain-derived neurotrophic factor (BDNF) expression. Isobavachalcone and bavachinin from PF also could improve learning and memory by inhibiting Ab42 aggregation. The JS extract could enhance the acetylcholine, acetylcholine transferase activity, and suppress AChE activity to prevent and treat AD. Allicin could effectively improve spatial learning and memory function in AD mice. However, the effects of QEF and its different compatibilities on learning and memory have rarely been reported.

In this paper, the effects of different compatibilities of QEF on the scopolamine-induced learning and memory impairment in mice were evaluated. The results demonstrated the mechanisms and compatible regularity of QEF for improving learning and memory.

Materials and Methods

Animals
20 ± 2 g male Kunming mice were obtained from Experimental Animal Center of Lanzhou University (Lanzhou, China) (Certificate of Conformity: SCXK (Gan) 2013-0002). All animals were housed in an air-conditioned room at temperature of 22°C ± 2°C and a relative humidity of 50% ± 10% with a 12 h dark-light cycle (light on from 7:00 to 19:00). All the animals were allowed to have free access to water and food. All experimental procedures were conducted in accordance with the guidelines for the use of experimental animals and approved by the Institutional Review Committee on Animal Care.

Chemicals and reagents
EC, PF, JS, and GR were purchased from the Sichuan Xinghehua TCM Electuary Co., Ltd. (Sichuan, China) and identified by Prof. Li-Hong Wu, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine. Scopolamine hydrobromide (Scop) was obtained from Shanghai Harvest Pharmaceutical Co., Ltd. Donepezil hydrochloride was obtained from Eisai (China) pharmaceutical Co., Ltd. The assay kit for AChE was purchased from the Nanjing Jiancheng Biotech Company. Rabbit polyclonal anti-BDNF antibody was purchased from Abcam, UK. Other chemicals were all analytical reagents.

Preparation of the extracts of Qing’e formula and its different compatibilities
The extracts of QEF and its different compatibilities were obtained as follows: (a) EC; (b) EC + PF; (c) EC + JS; (d) EC + GR; (e) EC + PF + JS + GR (QF); and (f) PF. Six different compatibilities of QEF were infiltrated with 8-fold mass of ethanol-water (75:25, v/v), respectively, and then extracted under reflux for three times, 3 h and each time, and filtrated with doubled absorbent gauze after each extraction. The six groups of the combined filtrate were evaporated to dryness, and the residues were reconstituted in 5% ethanol-water, respectively.

Experimental procedures
The random number table was used to divide the experimental mice into nine groups: (1) Control, (2) Model, (3) Positive, (4) EC, (5) EC + PF, (6) EC + JS, (7) EC + GR, (8) QF, and (9) PF, 15 mice in each group. The control group and model group were orally administered with the same dose of water, the positive group was orally administered with donepezil (1 mg/kg body weight), and other groups were orally administered with corresponding physic extracts (2 g/kg body weight), consecutively for 21 days. The control group was injected intraperitoneally (i.p.) with saline while the other eight groups were administered with Scop (2 mg/kg i.p.) before the behavioral experiments.

Step-down test
The inhibitory avoidance training apparatus was composed of a transparent box (32 cm × 22 cm × 33 cm) with a stainless-steel grid floor and an elevated rubber platform (4.5 cm in diameter and 4.5 cm in height) on the left side wall of the training box apparatus. Its floor was made of parallel 0.1 cm caliber stainless steel bars spaced 0.5 cm apart. The step-down test was carried out in 2 days including training and retention sessions. On the first training day, mice were allowed to adapt for 3 min in the box. After 3 min, electric shocks were delivered and mice jumped on the platform to avoid stimulation. If the animals stepped down from the platform (error trial), they were exposed to an electric foot shock (36 V, AC). After 24 h, latency was assessed again and recorded as the learning grade (latency), which was taken as a measure of memory retention.
Donepezil (1 mg/kg, p. o.) and the extracts of different compatibilities of QEF (2 g/kg, p. o.) or vehicle (the same volume of water) solution were administered to mice 40 min before the acquisition trials. Each acquisition trial was carried out 10 min after a single Scop treatment (2 mg/kg, i.p.).

**Morris water maze task**

The apparatus of MWM task consisted of a circular pool (40 cm in height and 80 cm in diameter) filled with water at 23°C ± 2°C. Leukophyll was added to the pool water to make it opaque. A clear plexiglas platform (10 cm in diameter × 17.5 cm in height) was submerged 1 cm below the water surface. The pool was divided into four equal quadrants and each quadrant was marked by a different visual cue. The platform was randomly placed in one quadrant during the experiment. The whole experiment lasted 6 days. On the 1st day, mice were allowed a 120 s habituation session in the pool without the platform. In the following 4 days, each mouse received four 120 s learning trials with the platform, with 60 s resting periods between trials. For each learning trial, mice were placed into the water facing the pool wall at one of four points of entry. The escape latency, the time required to locate the submerged platform, was recorded for each trial. If the mice were unable to locate the platform within 120 s, it was led to the platform and allowed to rest for 60 s. The escape latency in these cases was recorded as 120 s. After the completion of the learning trials, the platform was removed from the pool. The percentage of residence time and the percentage of swimming distance during platform quadrant were recorded, which would be the detection index of spatial memory.

Drug administration was identical to that of the step-down test.

**Acetylcholinesterase activity assay**

After the behavioral tests, the mice were sacrificed, and the hippocampus was carefully stripped from cerebral cortex and washed with ice-cold normal saline before it was dried with filter paper and weighed. The clean hippocampus was snap frozen in refrigerator and stored at −80°C until analysis. The hippocampus was rapidly dissected and homogenized in ice-cold normal saline to make 10% tissue homogenate that was followed with centrifuge at 3000 rpm for 15 min at 4°C. The supernatant was used for determination of AChE. The AChE activity was measured according to the instructions of the assay kit.

**Immunohistochemical staining**

Following the behavioral test, mice were deeply anesthetized with 0.4% pentobarbital sodium (10 mg/kg body weight), then the hippocampus was harvested on an ice board and fixed in a bottle with 4% paraformaldehyde solution for 24 h at 4°C. Hippocampus was embedded in paraffin wax and then cut into sagittal slices of 8 μm thickness by vibratome. Afterward, sections of every group were dewaxed and rehydrated. Next, sections were boiled in 0.01 mol citric acid buffer for 10 min. After cooling, these sections were incubated in 3% H2O2 for 5 min and then in 10% goat serum in PBS for 10 min. The sections were then incubated with rabbit anti-BDNF (1:500) overnight at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG and streptavidin-peroxidase complex. Sections were visualized by a reaction to 3,3'-diaminobenzidine tetrachloride kit (DAB, ZSGB-BIO, China) followed by dehydration and mounting on gelatin-coated slides.

**Statistical analysis**

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All data were expressed as (mean ± SD) a nonparametric test (Kruskal–Wallis test) was used to evaluate the difference between all groups. P < 0.05 was considered statistically significant.

**Results**

**Effects of different compatibilities of Qing’e formula on Scop-induced learning and memory impairment in the step-down test**

Learning and memory impairments in Scop-treated and control animals were evaluated using the step-down test [Figure 1a]. As shown in Figure 1b, the step-down latency of model group mice in the step-down test was significantly shorter than that of control group mice (P < 0.01). Figure 1b showed that the short step-down latency by Scop was significantly reversed after orally administered with donepezil, the extracts of EC, EC + PF, EC + GR, QF, and PF (P < 0.05 or P < 0.01). There was no distinct difference between the model group and the EC + JS treatment group. Of note, the efficacy of QF
Effects of different compatibilities of Qing’e formula on Scop-induced learning and memory impairment in the Morris water maze task

Spatial memory performance was assessed over successive 4 days using the hidden platform [Figure 2a], and a probe trial was conducted with no platform on the 6th day. In the navigation test, the model group exhibited longer escape latency than the control group [Figure 2b, P < 0.01]. Compared with model group, donepezil and the extracts of EC, EC + PF, EC + JS, EC + GR, QF, and PF significantly shortened the escape latency of Scop-treated mice in varying degrees [Figure 2b, P < 0.05 or P < 0.01]. Interestingly, the escape latency of QF group was less than that of other compatibility groups, but only the escape latency of EC + JS group had statistical significance compared with the QF group [Figure 2b, P < 0.05].

In the probe test, the residence time percent and swimming distance percent in the target quadrant of model group were significantly less than control group [Figure 2c and d, P < 0.01]. At the same time, donepezil and the extracts of EC, EC + PF, EC + JS, EC + GR, QF, PF not only dramatically increased the percentage of residence time [Figure 2c, P < 0.05 or P < 0.01] but also rose the percentage of swimming distance by comparison with model group [Figure 2d, P < 0.05 or P < 0.01]. Interestingly, the percentage of residence time and the percentage of swimming distance of QF group were greater than that of other compatibility groups, but only the percentage of residence time and the percentage of swimming distance of EC and EC + JS groups had statistical significance compared with the QF group [Figure 2c and d, P < 0.05 or P < 0.01].

Effects of different compatibilities of Qing’e formula on the acetylcholinesterase activity

As shown in Table 1, the activity of AChE in model group mice significantly increased in comparison with control group mice (P < 0.01). Donepezil and the extracts of EC, EC + PF, EC + JS, EC + GR, QF, PF significantly inhibited AChE activity in the hippocampus by comparing with the model group (P < 0.01). Simultaneously, distinct differences were observed between the QF group and other compatibilities groups (P < 0.01), that is to say, QF could show the highest level of improvement by inhibiting AChE activity.

Effects of different compatibilities of Qing’e formula on the expression of brain-derived factor

The hippocampus of control group had more positive neurons of BDNF, and the positive expression stained darker [Figure 3a], while the positive expression stained lighter and looser in the model group [Figure 3b]. Meanwhile, the positive expression of BDNF increased in donepezil group and different compatibilities of QEF groups by comparison with model group [Figure 3c-i and Table 1]. Moreover, the positive expression of BDNF in QF group was higher than that of other treatment groups except the EC + PF group [Table 1].

Discussion

As we all known, scopolamine is a nonselective centrally acting muscarinic receptor antagonist and impairs learning and memory in both rodents and humans.[21,22] This experimental model of memory impairment has been extensively used in

Figure 2: The effects of different compatibility of Qing’e formula on the Scop-induced learning and memory impairment in Morris water-maze task. (a) The apparatus for Morris watermaze; (b) The escape latency time to find the submerged platform; (c) The residence time percent; (d) swimming distance percent during platform quadrant. *P < 0.05, **P < 0.01 versus control group; *P < 0.05, **P < 0.01 versus model group; ΔP < 0.05, ΔΔP < 0.01 versus QF group
Qing’e formula improve learning and memory disorders

## Table 1: The effects of different compatibilities of Qing’e formula on acetylcholinesterase activity and brain-derived neurotrophic factor expression in hippocampus (mean ± standard deviation)

<table>
<thead>
<tr>
<th>Group</th>
<th>AChE (U/mg protein)</th>
<th>BDNF</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.41±0.11</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>Model</td>
<td>1.06±0.19**</td>
<td>0.18±0.01**</td>
</tr>
<tr>
<td>Donepezil</td>
<td>0.48±0.14**</td>
<td>0.23±0.01**</td>
</tr>
<tr>
<td>EC</td>
<td>0.66±0.21***</td>
<td>0.22±0.05*</td>
</tr>
<tr>
<td>EC + PF</td>
<td>0.53±0.24**</td>
<td>0.26±0.01**</td>
</tr>
<tr>
<td>EC + JS</td>
<td>0.55±0.10*</td>
<td>0.18±0.01**</td>
</tr>
<tr>
<td>EC + GR</td>
<td>0.56±0.08***</td>
<td>0.21±0.01*</td>
</tr>
<tr>
<td>QF</td>
<td>0.31±0.08**</td>
<td>0.25±0.02**</td>
</tr>
<tr>
<td>PF</td>
<td>0.64±0.08***</td>
<td>0.19±0.02**</td>
</tr>
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</table>


To sum up, different compatibilities of QEF improved learning and memory deficits in scopolamine-induced mouse models through suppressing AChE activity and increasing BDNF expression. Among all compatibility groups, the group that was treated with QF extract showed the highest level of improvement. Therefore, this paper clarified compatibility rationality of QEF and provided a new possible drug in AD treatment. In addition, our study also found that the extract of PF alone could significantly improve the learning and memory ability of model mice, which was consistent with the experiment of Chen,[10] but its possible mechanism and active ingredients still need further study.

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Conflicts of interest
There are no conflicts of interest.

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