Study of Ameliorating Effects of Ethanolic Extract of *Centella asiatica* on Learning and Memory Deficit in Animal Models

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**Objective:** The present study investigated the effect of *Centella asiatica* ethanolic extract (CE) on learning and memory impairment induced by either transient bilateral common carotid arteries occlusion (T2VO) or an intraperitoneal injection of scopolamine in mice.

**Material and Method:** CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) were administered to learning and memory impaired mice once daily for 8 consecutive days. Learning and memory performance were evaluated by Morris water maze (MWM) and step-down passive avoidance (PA) test. Changes in malondialdehyde (MDA) levels in the brain were determined by lipid peroxidation assay.

**Results:** T2VO mice exhibited learning and memory impairment in the MWM and PA tests. Treatment with CE ameliorated the learning and memory impairment of T2VO mice. Furthermore, CE significantly reduced MDA level in the brain of T2VO mice. On the other hand, administration of CE did not attenuate learning and memory impairment induced by scopolamine in mice.

**Conclusion:** The present study demonstrated ameliorating effect of CE on learning and memory impairment in T2VO mice. Furthermore, it is likely that the positive effect of CE observed could be, at least partly, accounted by its antioxidative property. Thus, CE might be beneficial for memory impairment in which oxidative stress is an underlying cause.

**Keywords:** *Centella asiatica*, Learning and memory, Transient bilateral common carotid arteries occlusion (T2VO), Scopolamine

Alzheimer’s disease is an age-related neurodegenerative disorder which is the most common cause of dementia in the elderly. The pathological features that have been identified in the central nervous system (CNS) in Alzheimer’s disease are oxidative and inflammatory processes. Although a number of drugs have been approved for use, they have been shown to produce side effects and yield relative modest benefits. To overcome these limitations of current therapeutics for Alzheimer’s disease, extensive research and development are underway to identify drugs that are effective and free of undesirable side effects. Recently, natural medicines have gained considerable attention as alternative candidates for Alzheimer’s disease therapy.

*Centella asiatica* is a herb which have been used for centuries in Ayurvedic medicine as one of the main ingredients in Ayurvedic formulation to retard ageing and to prevent dementia. In traditional Chinese medicine, *C. asiatica* has been used for combating physical and mental exhaustion. Preliminary studies on the pro-cognitive effect of *C. asiatica* have demonstrated that extract of this herb enhances memory retention in rodents. Phytochemical analyses have shown that *C. asiatica* contains triterpenoid saponins, flavonoids, phytosterols and other components. Asiaticoside, the most abundant triterpene saponin, has been reported as a cognitive enhancer useful in treating dementia. However, there has been no report...
on the effect of *C. asiatica* on the animal model of transient cerebral ischemia or scopolamine-induced learning memory deficit. Therefore, the present study aimed to explore the effect of ethanolic extract of *C. asiatica* (CE) on transient bilateral common carotid arteries occlusion (T2VO) or scopolamine-induced learning and memory impairment in mice. Since Alzheimer’s disease have been associated with increased oxidative stress\(^8\), the effect of CE on marker of brain oxidative stress namely malondialdehyde (MDA) was also conducted.

**Material and Method**

**Plant materials and preparation of the extract**

*C. asiatica* plants were procured from local supplier in Nonthaburee province, Thailand. The leaves were washed thoroughly, sun dried and ground into powder. The dried plant (7.1 kg) was extracted with 95% ethanol for 3 days and filtered. The pooled extract was concentrated under reduced pressure by rotary flash evaporator to obtain syrupy mass which was then evaporated to dryness on water bath. The yield of the crude CE was 12.68 % (W/W).

**Chemicals**

All chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, Mo, USA).

**Animals**

Eight-week-old male ICR mice (National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand) weighing 30-35 g were housed, 8 mice per cage, under standard conditions (ambient temperature 25±2°C, humidity 60-70%, 12-h light/dark cycle (lights on at 7:00 h)) and allowed free access to both food pellets and water. They were allowed to acclimatize to these housing conditions for 1 week prior to the experiments in which they were divided into experimental groups of 8 animals in each. All behavioral experiments were carried out between 9:00 and 17:00 h. The experimental protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

**Induction of learning and memory impairment in mice**

Learning and memory impairment was induced by T2VO or an administration of scopolamine. T2VO was performed by method described previously by Xu et al\(^9\). Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). A ventral midline incision of the neck was made. The common carotid arteries were exposed, carefully separated from surrounding tissue and vagus nerve and then occluded by arterial clips. While the arteries were clamped, blood (0.3 ml) was withdrawn by cutting off the tip of the tail. After 20 min, the arterial clips were removed and the incision was closed. Sham-operated mice were subjected to the same procedure but without carotid clamping and bleeding. Animals were allowed to recovery for 24 h before behavioral studies. For scopolamine-induced learning and memory impairment, mice were intraperitoneally injected with scopolamine (0.5 mg/kg, dissolved in normal saline) 30 min before each behavioral test\(^10\).

**Drug administration**

CA was dissolved with 15% Tween 20 in distilled water and administered orally by gavage. Vehicle was given to sham operated mice and CE (100, 300, 1,000 or 1,500 mg/kg) was given to T2VO-induced learning and memory deficit mice at 1 h before the onset of behavioral test at every consecutive day (8 days).

In the scopolamine-induced learning and memory impairment study, CE was given 40 min before the onset of behavioral test at every consecutive day (8 days). Vehicle was administered to the control group using the same time schedule.

**Morris water maze test**

The procedure used was slightly modified from the one that described by Morris\(^11\). The Morris water maze (MWM) is a black circular pool with a diameter and height of 70 and 23 cm, respectively. The pool was filled to a depth of 13 cm with water (25±1°C). The pool was divided into four quadrants and placed in a room with various visual cues in fixed positions. A black platform (6 cm in diameter) was placed at the middle of one quadrant of the pool and submerged 1 cm below the water surface. Daily swimming consisted of four trial sessions in which the mice were placed in the water facing the pool wall from four different starting points. Mice were allowed 60 sec to locate the platform in each trial session and 30 sec to rest between the trial sessions. This was conducted for 8 consecutive days. The time taken to find the platform in each trial session was then recorded and averaged as an escape latency of each animal per day. Two days before these days were considered as training days in which the animal that could not locate the platform would be guided by researcher onto the platform and allowed to remain on
it for 10 sec. The mice that failed to locate the platform after four trial sessions were excluded from the experiments.

**Step-down passive avoidance test**

A step-down passive avoidance (PA) test was examined in a Plexiglas chamber (35x23x20 cm³) with a stainless steel grid floor (3 mm stainless steel rods spaced 11 mm apart) attached to a shock generator (1 Hz, 1 ms, 36 V DC) as previously described[12]. A wooden platform (5 cm in diameter) was placed in one corner of the chamber serving as a shock free zone. The PA test consisted of two trials; acquisition and retention trial. The acquisition trial was performed on the next day after the last MWM test. In the acquisition trial, the mouse was placed in a chamber without an electric foot shock for 3 min, and then the electric shock was delivered through the stainless steel rods for 5 min. The mouse could escape the electric shock by moving to the platform. Twenty-four hours after the acquisition trial, the mouse was placed on the platform in a chamber for a retention trial, and the electric shock was delivered. The initial step-down latency and the number of errors were recorded for 5 min. Initial step-down latency was the time that elapsed until the mouse stepped down from the platform. If the mice did not step down from the platform within 300 sec, the initial step-down latency was recorded as 300 sec. Upon electric shock, the mice would escape from the grid floor back to the platform and the number of error was counted whenever the animal stepped down from the platform.

**Locomotor activity test**

Spontaneous locomotor activity was evaluated using an activity cage (model 7430, Ugo Basile, Comerio, Italy). The apparatus consisted of a Plexiglas chamber (35x23x20 cm³) with a stainless steel grid floor (3 mm stainless steel rods spaced 11 mm apart) connected to the circuit of counting unit. On the day after retention trial of the PA test, the mouse was placed in the activity cage, and locomotion was immediately recorded for 5 min.

**Lipid peroxidation assay**

Following the locomotor activity test, the animals were sacrificed by decapitation and the brains were quickly removed, cleaned with ice-cold saline and stored at -80°C. Brain tissue samples were thawed and homogenized with ice-cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates were used to determine the MDA content which is a marker of lipid peroxidation by the method previously described by Gupta et al[13]. Briefly, the reagents including 1.5 ml of acetic acid pH 3.5 (20%), 1.5 ml of thiobarbituric acid (0.8%) and 0.2 ml of sodium dodecyl sulphate (8.1%) were added to 0.1 ml of brain tissue samples and then heated at 100°C for 60 min. The mixture was cooled under tap water then 5 ml of n-butanol/pyridine (15:1) and 1 ml of distilled water was added and vortexed vigorously. After centrifugation at 2,500 rpm for 20 min, the organic layer was separated. The supernatant was collected and the absorbance was measured at 532 nm by spectrophotometry. The concentration of MDA was expressed as nmol/g tissue.

**Statistical analysis**

Results are expressed as mean ± SEM. Data obtained from behavioral and biochemical study were evaluated using Student’s t-test or one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. A p-value <0.05 was considered statistically significant.

**Results**

**Effects of CE on memory impairment induced by T2VO**

**Morris water maze test**

The effect of CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) on spatial learning and memory impairment was evaluated using the MWM test. The MWM test is a behavioral procedure widely used in behavioral neuroscience to study spatial learning and memory[14]. In this test, mice are required to learn to locate the hidden platform, and the latency to locate the platform serves as a measure to spatial learning and memory performance. As shown in Fig. 1, mice subjected to T2VO exhibited longer latencies (44.88±0.38 sec on day 1) to locate the platform than sham-operated mice (23.91±3.17 sec on day 1) throughout the testing trial indicating impairment of learning and memory. On day 5, the escape latency of the T2VO group was 36.38±2.08 sec while it was 9.66±1.81 sec in sham-operated group. Orally given CE, 100, 300, 1,000 or 1,500 mg/kg, significantly attenuated the T2VO-induced deficit in learning and memory. As shown in Fig. 1, mice subjected to T2VO exhibited longer latencies (44.88±0.38 sec on day 1) to locate the platform than sham-operated mice (23.91±3.17 sec on day 1) throughout the testing trial indicating impairment of learning and memory. On day 5, the escape latency of the T2VO group was 36.38±2.08 sec while it was 9.66±1.81 sec in sham-operated group. Orally given CE, 100, 300, 1,000 or 1,500 mg/kg, significantly attenuated the T2VO-induced deficit in learning and memory. The escape latency on day 5 was found to be 14.44±0.97, 11.88±1.77, 10.44±0.76, and 8.09±0.61 sec for respective groups of CE.

**Step-down passive avoidance test**

As shown in Fig. 2A, the initial step-down latency of T2VO mice (46.13±1.56 sec) was significantly...
shorter than that of the sham-operated mice (195.38 ± 3.78 sec). Administration of CE, 300, 1,000 or 1,500 mg/kg, significantly increased the initial step-down latency in T2VO mice to 70.75 ± 8.22, 160.38 ± 3.78 and 147.88 ± 2.68 sec, respectively. Similar results were accordingly observed in terms of the number of error in which CE significantly decreased an increment of number of error observed in T2VO mice (Fig. 2B).

**Spontaneous locomotor activity**

An assessment of locomotor activity revealed no significant difference between T2VO and sham-operated mice (Fig. 3). Moreover, CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) treatment had no effect on the spontaneous locomotor activity of T2VO mice (Fig. 3).

**Lipid peroxidation**

The brain MDA levels, an indicator of lipid peroxidation, of different experimental groups were shown in Fig. 4. The brain MDA levels of the T2VO mice (634.63 ± 19.09 nmol/g tissue) were significantly higher than that of the sham-operated mice (201.88 ± 7.08 nmol/g tissue). The brain MDA levels of T2VO mice were significantly attenuated by CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) treatment (Fig. 4).

**Effects of CE on memory impairment induced by scopolamine**

**Morris water maze test**

The effect of CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) on memory impairment induced by scopolamine was shown in Fig. 5. In comparison to normal saline-treated mice, significant increase of the escape latency in MWM test was observed in mice receiving scopolamine, an anticholinergic drug used to induce experimental dementia(15). This effect was seen on the first day of testing trial and persisted throughout the testing period (Fig. 5). The escape latency on day 5 of normal saline-treated mice was 7.69 ± 1.03 sec whereas it was 39.53 ± 2.83 sec in scopolamine-treated mice. Administration of CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) to scopolamine treated-mice 40 min before the first testing trial of each day did not affect the impaired
Effects of CE on spontaneous locomotor activity of transient bilateral common carotid artery occlusion (T2VO)-induced memory deficit mice. Mice were orally treated with CE (100, 300, 1,000 and 1,500 mg/kg, p.o.) or vehicle (15% Tween 20) for 8 days. Final administration was performed 60 minutes before the test. Locomotor activity was measured for 5 min. Each datum presents the mean ± SEM for n = 8. No significant differences were detected.

Effects of CE on brain MDA levels of transient bilateral common carotid artery occlusion (T2VO)-induced memory deficit mice. Following the locomotor activity test, the mice were sacrificed and the brains were collected. MDA level was determined using lipid peroxidation assay. Each datum presents the mean ± SEM for n = 8. *p<0.001 versus vehicle-treated sham group (Student’s t-test), **p<0.001 versus vehicle-treated T2VO group (one-way ANOVA followed by Tukey’s post-hoc test).

**Step-down passive avoidance test**

In the PA test, scopolamine significantly shortened the initial step-down latency and increased the number of error (Fig. 6A and B). CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) treatment had no effect on the step-down latency and the number of error of scopolamine-treated mice (Fig. 6A and B).

**Lipid peroxidation**

As shown in Fig. 8, no significant differences in the brain MDA levels were found between scopolamine- and saline-treated mice. Treatment with CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) showed no significant effect on the brain MDA levels of scopolamine-treated mice (Fig. 8).

**Discussion and Conclusion**

In the present study, CE was evaluated in mice for its effect on learning and memory impairment induced by T2VO or an intraperitoneal injection of scopolamine. In line with previous report(16) that animal models of T2VO exhibited progressive spatial cognitive deficits, T2VO adversely affected learning and memory performances assessed by MWM and PA tests, the tests commonly used to screen for changes in learning and memory due to damage of the hippocampus(17). The finding that T2VO produced no effect on spontaneous locomotor activity but significantly increased escape latencies in the MWM test and produced a decrease in initial step-down latency of PA test agrees well with the fact that hippocampus is extremely vulnerable to cerebral ischemia. Additionally,
Oxidative stress is important in pathogenesis of Alzheimer’s disease, in clinical trials, a potential antioxidant, idebenone, showed a clear dose-related anti-dementia activity in Alzheimer’s disease (22). With regards to natural antioxidant enzymes or agents which are capable of augmenting the functions of these enzymes, several reports have shown that the natural remedies possessing antioxidant property, for example *Ginkgo biloba*, *Celastrus paniculatus*, *Clitoria ternatea*, *Lycoris radiate*, *Polygala tenuifolia* and *Salvia miltiorrhiza* could be relevant to the treatment of Alzheimer’s disease (2,20). Accordingly, oral administration of CE at the doses of 100, 300, 1,000 and 1,500 mg/kg showed an improvement in learning and memory deficits as evidenced by decreased escape latency (A) and number of error (B) were measured for 5 min. Each datum presents the mean ± SEM for n = 8. *p<0.001 versus vehicle-treated sham group (Student’s t-test).

The findings observed clearly indicated that poor performances in MWM and PA tests of T2VO group could not be explained by deficit or impairment of spontaneous locomotor activity. Furthermore, lipid peroxidation, one of the pathways underlying neuronal damage and cell death in Alzheimer’s disease (19) was found to be significantly increased exclusively in T2VO model but not in scopolamine treated mice. It is therefore suggestive that impairment of learning and memory in T2VO model is likely liked to an increase of MDA.

As the level of brain lipid peroxide in Alzheimer’s disease patient is higher than that of normal (19), MDA, marker of lipid peroxidation has been used as an index of oxidative stress in Alzheimer’s disease. A growing number of studies demonstrate that natural extracts and phytochemicals have a positive impact on brain aging (2,20). Moreover, clinical data suggest that nutritional antioxidants might exert some protective effect against Alzheimer’s disease (21).

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latency in the MWM and increased initial step-down latency and decreased number of errors in the PA test. Furthermore, an increase of cerebral MDA induced by T2VO was significantly reduced but not totally abolished by the administration of CE suggesting that ameliorating effect of CE on impairment of learning and memory observed in behavioral study could be partly accounted by antioxidant property of CE. Though antioxidant properties of C. asiatica have been similarly proposed to underlie positive effect on learning and memory in normal rats some other mechanisms possibly be involved and warranted further investigation.

Scopolamine is a tropane alkaloid drug with anticholinergic property, acting as a competitive antagonist at muscarinic cholinergic receptors. It has been shown to impair memory in humans and experimental animals to mimic the cognitive deficits found in Alzheimer’s dementia. Although pathogenesis of Alzheimer’s disease remains to be fully defined, several pharmacological strategies for treatment of Alzheimer’s disease are under active investigation. Cholinergic therapy that is designed to increase cholinergic functions and antioxidant are most attractive approach for treatment of Alzheimer’s disease. Tacrine is the first acetylcholine esterase inhibitor that has been approved for treatment of Alzheimer’s disease. In animal model, tacrine improved impairment of performance in the PA and the MWM tests. Furthermore, tacrine produced an ameliorative effect on eight-arm radial maze test deficit caused by T2VO in rats, and raised the levels of extracellular acetylcholine in the brain.

In the present study, an intraperitoneal administration of scopolamine induced a deficit of learning and memory performances in MWM as well as in the PA tests. However, unlike T2VO model, impairment of learning and memory induced by scopolamine was not associated with an increase of cerebral MDA. Interestingly, CE (100, 300, 1,000 or 1,500 mg/kg, p.o.) which has been found to improve learning and memory deficit in T2VO showed no effect on scopolamine-induced learning and memory deficit. Cerebral MDA level was neither affected by scopolamine or CE. Rivastigmine, an anti-cholinesterase drug currently used for Alzheimer’s disease, has been reported that in parallel with the ability to inhibit cholinesterase in cortex and hippocampus, it could improve scopolamine-induced deficit in the MWM and the PA tests. Therefore, the negative effect of CE on learning and memory function in mice receiving scopolamine suggests the lack of its effect on cholinergic system.

In conclusion, by means of MWM and the PA tests, the present study demonstrated the beneficial effects of CE on learning and memory impairment exclusively in T2VO mice but not that induced by scopolamine. Based on the finding that T2VO but not scopolamine significantly increased the lipid peroxidation which could be partly ameliorated by CE, therefore, it is suggestive that anti-oxidative property of CE could, at least partly, contributed to its positive effect on memory deficit in T2VO mice. This may explain also the lack of effect of CE on memory impairment induced by scopolamine in which oxidative stress was not its feature. Thus, CE might be beneficial for memory impairment in Alzheimer’s disease which oxidative stress is an underlying cause. Further study is needed to identify the active components underlying positive effect of CE on learning and memory deficit associated with cerebral ischemia.

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Potential conflicts of interest
None.

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การศึกษาผลคุณภาพชีวิตในภาวะเรื้อรังและความจำของสารสังเคราะห์ออกจากร่างในโมเลกุลตัวคลอง

ศาสตราจารย์ คุณภัทร วันนาบุญ, อุรุร วัฒน์มั่นรุ่ง, นพ. วชิรพร สมภู และ สมศักดิ์ ตันศิริ

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