Ameliorative effects of *Thunbergia laurifolia* on cognitive deficit and depression in olfactory bulbectomized mice

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1. Introduction

Recently, dementia has been one of the world's important public health problems. Alzheimer's disease (AD) is the most common form of dementia, a neurodegenerative disease characterized by progressive memory loss and cognitive impairment [1]. AD is pathologically characterized by extracellular deposition of senile plaques; formation of intracellular neurofibrillary tangles; and lesions of cholinergic neurons together with synaptic alterations in cerebral cortex, hippocampus, and other brain regions[2]. However, current approved treatments including cholinesterase inhibitors and agonists of *N*-methyl-D-aspartate receptors (NMDA) do not terminate the progression of the disease, and have provided marginal therapeutic benefits [3]. Therefore, it is very essential to discover novel and effective medications for AD.

Thunbergia laulifolia Lindl., commonly known as Rang Jurd (RJ), belongs to the botanical family of Acanthaceae. The aqueous preparations of leaves and root have been used in Thai traditional medicine as anti-inflammatory and antipyretic agents as well as antidote for detoxification of poison including insecticide, ethyl alcohol, arsenic and strychnine [4, 5]. Preparation of the extracts of dried TL leaves with boiling water was found to contain apigenin and its glycosides, phenolic acids such as caffeic acid, gallic acid and protocatechuic acid [6]. In mice, RJ leaf extract alleviated adverse effects of lead on learning deficit and memory loss, evaluated with water maze swimming test [7].

Several studies suggest that impairment of the olfactory system leads to impaired memory and cognitive function in rodent. For example, impaired performance on learning and memory tasks, such as passive avoidance, modified Y-maze and object recognition, was observed in olfactory bulbectomized (OBX) mice and rats [8-10]. In addition, elevated levels of β-amyloid peptide [11] and degeneration of cholinergic neurons [12] are observed in the brain of OBX mice. Interestingly, the impairment of the olfactory system is not only observed in the early stages of Alzheimer's disease (AD) [13, 14], but also in a transgenic AD model of mice, demonstrating that OBX provides a beneficial animal model of AD that is independent from transgenic animal models.

2. Materials and Methods

2.1 Animals

The overall study was conducted according to the experimental protocols described in Fig. 1 and 2. Male ddY mice (Japan SLC Inc., Shizuoka, Japan) were obtained at the age of 9 weeks old and housed with a 12-h light/dark cycle (lights on: 07:30–19:30) at 22 ± 1 ℃. Food and water were available ad libitum. The animals were habituated to the laboratory animal room for at least 1 week before surgery. The behavioral experiments were performed during the light phase from 9:00 to 18:00. The present studies were conducted in accordance with the Guiding Principles (NIH publication #85-23, revised in 1985) for the Care and Use of Animals and were approved by the Institutional Animal Use and Care Committee in the University of Toyama.

2.2 Plant extract preparation

The leaves of *Thunbergia laulifolia* were collected from Wangnamyen district, Sa kaeo province, Thailand in 2011. The plant samples were identified by Mr. Pinit Chinsoi, Pharmacist, Wangnamyen Hospital, Sa Kaeo province, Thailand. The voucher specimens were deposited at Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, Thailand (RJ01001). The leaves were cleaned, dried in a hot air oven (60℃) for 6 h, and powdered with electronic mill (20 mesh sieve) then were boiled with distilled water (1:10 w/v) for 8 h and filtered. The filtrate was taken to dryness by lyophilization to yield dried leaf decoction extracts (RJ).

2.3 Surgical operation

OBX of mice was conducted according to a previous report. Briefly, the mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and fixed on stereotactic instruments (Narishige, Tokyo, Japan). The skull covering the bulbs was exposed by skin incision, 1% lidocaine solution was used as local anesthetic then a 1mm burr hole was drilled. The bilateral bulbs were aspired through a syringe and the cavity of the bulbs was filled with hemostatic gelatin sponge. After completing behavioral studies, all the animals were sacrificed and the operated lesion was verified visually. The data from animals with less than 70% of removal or with not intact cortex was excluded from data analysis. Sham operation was performed in a similar way without removal of the bulb. At the end of experiments, the olfactory bulbs of sham group mice were confirmed to be intact.

2.4 Drug administration

Tacrine (THA, 2.5 mg/kg, i.p.) was kindly supplied by Eisai Co. Ltd. (Tokyo, Japan) and Imipramine (IM, 10 mg/kg, i.p.) were separately dissolved in 0.9% normal saline solution and intraperitoneally administered. Sham group of mice and OBX control group mice were orally administered water. Leaf extract of *T. laulifolia* was dissolved in water and given per orally at daily doses of 250–500 mg/kg. On a behavioral testing day, either THA or RJ was administered 1 h before the testing.

2.5 Behavioral study

2.5.1 Locomotor activity

Ten days after the surgical operation, the animals underwent administration of test drugs once daily for a week. One hour after the last administration, locomotor activity of each animal in the open field (35x 35x50 cm) was video-recorded for later analysis. The activity during a 10-min observation period was analyzed using Smart® system (PanLab, S.L., Barcelona, Spain). The total distance exploring the arena was used to determine the locomotor activity.

2.5.2 Modified Y-maze test

Forty mice were randomly divided into four groups of ten mice. Each group of mice was orally administrated with distilled water as a control group, tacrine (2.5 mg/kg, i.p.) was used as a standard drug, and *T. laulifolia* leaf extract in 2 different concentrations of 250 and 500 mg/kg, p.o., respectively. A modified version of the Y-maze test has been reported by Yamada et al. [15]. The apparatus used for the modified Y-maze test consists of black polypropylene walls with 3 arms each 40cm long, 12cm wide at the top, 3 cm wide at the bottom, 18cm high. This test is a two-trial task with a sample phase trial and a test phase trial that are separated by an inter-trial interval. In the sample phase trial, each mouse was individually placed in the maze with one of the 3 arms closed. The animals were allowed to freely explore the other 2 arms for 5 min. Thirty min after the sample phase trial, the animal was again placed in the maze with all 3 arms opened, and allowed to explore the arms freely. The closed arm that is opened in the test phase trial is defined as the new arm. The animal behavior was video-recorded for later analysis. Percent time spent in the new arm and numbers of total arm entries were analyzed by Smart® system.

2.5.3 Novel object recognition test (ORT)

This test is based on the tendency of mice to discriminate a familiar from a new object. Mice were individually habituated to an open-field box (35x 35x50 cm) for 10 min on the day before the experiment. The ORT consists of a sample phase trial and a test phase trial. During the sample phase trial, two objects of the same material were placed in a symmetric position in the center of the chamber for 10 min. thirty minute after the sample phase trials, one of the objects was replaced by a novel object, and exploratory behavior was again analyzed for 5 min. After each session, objects were thoroughly cleaned with 70% ethanol to prevent odor recognition. Exploration of an object was defined as rearing on the object or sniffing it at a distance of less than 2 cm. Successful recognition of a previously explored object was reflected by preferential exploration of the novel object. Discrimination of spatial novelty was assessed by comparing the difference between time of exploration of the novel and familiar

2.5.4 Tail suspension test (TST)

Tail suspension test (TST) is a widely used model for assessing antidepressant effect. Mice

were subjected to the short-term inescapable stress of being suspended by the tail that will develop an immobile posture. Using another group of mice as indicated in protocol#2 (Fig 2), three mice were separately suspended 50 cm above the floor in a chamber by adhesive tape placed approximately 2 cm from the tip of the tail. Immobility time was recorded during a 8 min period using Smart® system.

2.6 Neurochemical study

2.6.1 Ex vivo measurement of cholinesterase activity in the brain

After completing the behavioral experiments, the OBX mice treated with daily administrations of tacrine and RJ were decapitated and the frontal cortices were dissected out and kept at −80 ◦C until use. Determination of cholinesterase activity was performed on the basis of the colorimetric method. Briefly, the frozen cortex was weighed and homogenized in 10-times volume of 0.1M phosphate buffer (pH 7.4) containing 1% Triton-X-100. After centrifugation at 15,000 × g at 4 ◦C for 20 min, the clear supernatants were collected and served as the enzyme source. Cholinesterase activity was determined in 96-well flat-bottom microplates. The reaction was started by adding 20 μl of 10mM 5,5´-dithiobis-(2-nitrobenzoic acid), 20 μl of 30mM acetylthiocholine, and 160 μl of phosphate buffer. The spectrophotometric absorption at 405 nm during a 3 min incubation period at 25 ◦C was quantitatively measured using a microplate reader (Sunrise Classic; TECAN Japan, Kawasaki) and expressed as nmol ACh hydrolyzed/min/mg tissue.

2.6.2 In vitro measurement of cholinesterase activity in the brain

Cholinesterase activity was determined in 96-well flat-bottom microplates using frontal cortex supernatants as the enzyme source. As same as the ex vivo experiment, the reaction was started by adding 20 μl of 10mM 5,5´-dithiobis-(2-nitrobenzoic acid), 20 μl of 30 mM acetylthiocholine, 50 μl of RJ or tacrine, and 160 μl of phosphate buffer. The spectrophotometric absorption at 405 nm during a 3 min incubation period at 25 ◦C was quantitatively measured using a microplate reader (Sunrise Classic; TECAN, Kawasaki, Japan). The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer without any inhibitor. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. Every experiment was done in triplicate.

2.6.3 Quantitative real-time polymerase chain reaction (PCR)

To analyze changes in expression levels of choline acetyltransferase (ChAT) and muscarinic M1 receptor mRNA in the brain, the animals were killed by decapitation after completing the behavioral studies. The brain was removed immediately and the hippocampus were dissected out and kept at − 80°C until use. Quantitative PCR was conducted as previously described [16]. Briefly, total RNA was extracted from the hippocampus using Sepazol® (Nacalai Tesque, Kyoto)

according to the manufacturer's instructions. First-strand cDNA synthesis was conducted using oligo (dT) primers and M-MLV Reverse Transcriptase® (Invitrogen, Rockville, MD, USA) in a total volume of 20 μl. The reaction was performed at 25°C for 10 min and heated at 37°C for 60 min and 98°C for 5 min before cooling to 4°C. DNA corresponding to the RNA was used as a template for real-time PCR. Quantitative realtime PCR was carried out using Fast SYBR Green Master Mix (Applied BioSystems, Foster City, CA, USA) in a StepOne Real-time PCR System (Applied BioSystems). The following primer sets used in this study were synthesized by Nippon EGT Co. (Toyama): ChAT mRNA (NM_009891): 5′-CCTGTACAAGCTTCTAGCTGTGAG-3′ (forward) and 5′-GTAGCTAAGCACACCAGAGATGAG-3′ (reverse); M1 mRNA (M16406): 5′ -ACTGTCTTGGCACCAGGAAA-3′ (forward) and 5′-TGCTAGGCCAATCATCAGAG-3′ (reverse) and β-actin mRNA (NM_007393): 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ (forward) and 5′ -ATGGAGCCACCGATCCACA-3′ (reverse). Melting curve analysis of each gene was performed every time after amplification was completed. Standard curves of the log concentration of each gene vs. cycle threshold were plotted to prove negative linear correlations.

2.7 Data analysis

The results were expressed as the mean ± SEM. The data obtained from the behavioral tests and neurochemical experiments were analyzed by pair and unpaired student's *t*-test or one-way analysis of variance (ANOVA) followed by a post hoc multiple comparison test (Dunnett's method) as appropriate. Differences of $P < 0.05$ were considered as significant. The analysis was conducted using SigmaStat® ver 3.5 (SYSTAT Software Inc., Richmond, CA, USA).

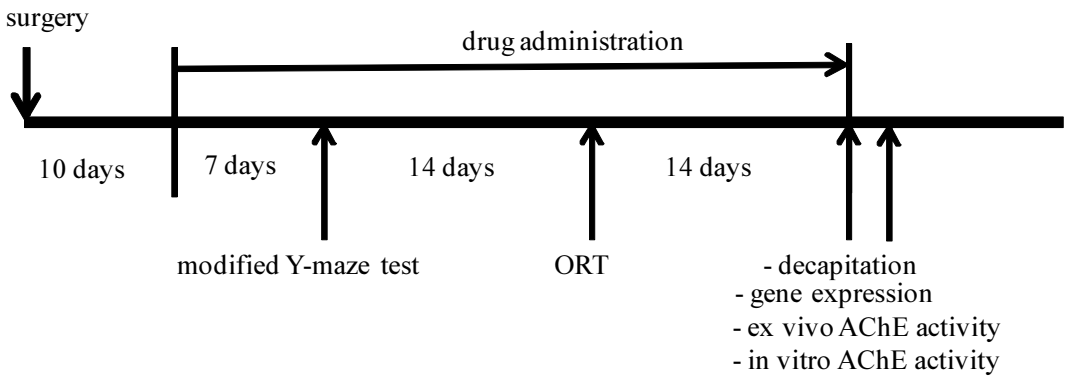

Fig 1. Schematic drawing of experimental schedule protocol#1. After one week of acclimatization, the ddY mice were randomly divided into 4 groups of 10 mice. All mice (except mice in sham group) were subjected for OBX surgery. Ten days after the surgery, the drug administration was started. Modified Y-maze and object recognition tests were performed 1 and 3 weeks after starting drug administration, respectively. Quantitative real-time polymerase chain reaction and neurochemical study were done after decapitation of all mice.

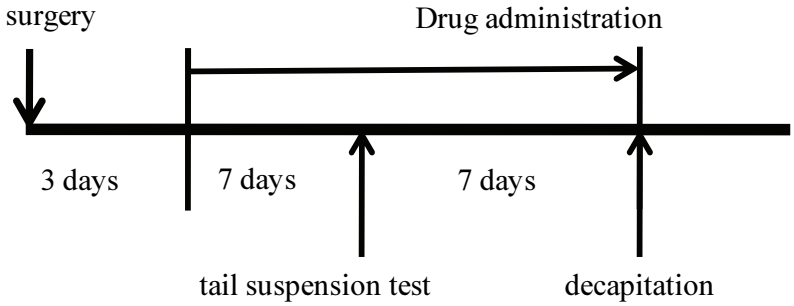

Fig 2. Schematic drawing of experimental schedule protocol #2. After one week of acclimatization, the ddY mice were randomly divided into 4 groups of 6 mice. All mice (except mice in sham group) were subjected for OBX surgery. Three days after the surgery, the drug administration was started. Tail suspension test was performed 7 days after starting drug administration.

Results

3.1 The effect of RJ and Tacrine on OBX-induced elevation of motor activity

Operation	Daily treatment	Locomotor activity (cm/10 min, mean ± S.E.M)
Sham	vehicle	5569.6 ± 293.0
OBX	vehicle	6640.2 ± 513.2
OBX	250 mg/kg (p.o.) RJ	6019.3 ± 237.7
OBX	500 mg/kg (p.o.) RJ	5918.8 ± 249.5
OBX	2.5 mg/kg (i.p.) tacrine	5679.8 ± 349.8

Table 1. Effect of RJ and tacrine on locomotor activity of OBX mice
The sham-operated or olfactory bulbectomized (OBX) mice received daily administration of vehicle (distilled water), tacrine or RJ according to protocol #1. Each datum represents the mean ± S.E.M. from 10 mice in each group.

As summarized in Table 1, there was no significantly difference in locomotor activity determined as total distance between vehicle-treated OBX and sham mice groups. Moreover, daily administration of tacrine (2.5 mg/kg, i.p.) or RJ (250 and 500 mg/kg, p.o.) for 1 week before the experiments showed no effect on locomotor activity.

3.2 RJ had no effect on OBX-induced spatial working memory deficits in the modified Y-maze test

As shown in Fig. 3, percentage time spent exploring the new arm of vehicle-treated sham mice (49.30 ± 2.19%) was significantly higher than the chance level of 33.09 ± 3.05% indicating a preference for new arms over the familiar arms. The tacrine-treated OBX mice(2.5 mg/kg, i.p.) spent significantly longer time visiting new arm than the vehicle-treated OBX group (50.26 ± 2.75),while RJ treated groups (250 and 500 mg/kg. p.o.) did not spend significantly longer

time visiting new arm than the vehicle-treated OBX group (40.12 ± 1.75% and 43.20 ± 2.41% , respectively).

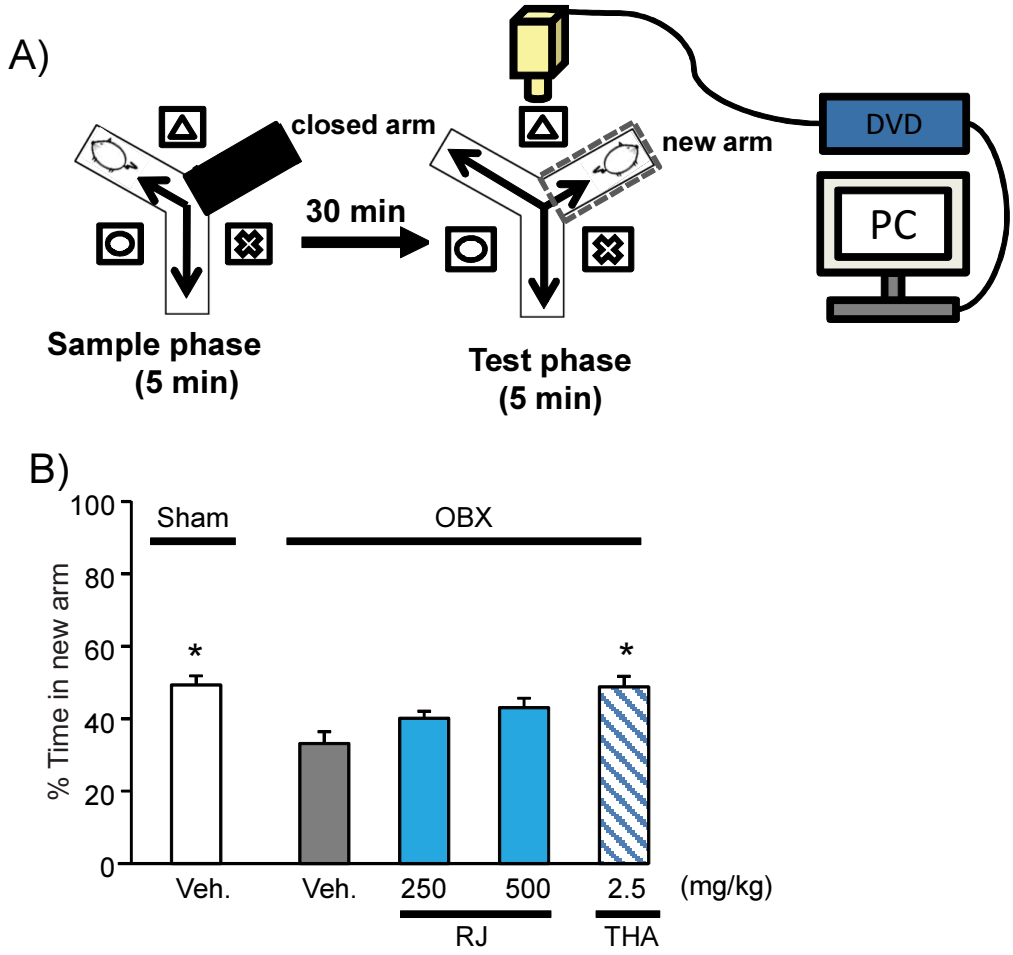

Fig. 3 Evaluation of the modified Y-maze test of OBX-induced spatial working memory deficit mice using tacrine or *T. laulifolia* leaf extract (RJ). A: Schematic illustration of an experimental protocol for the modified Y-maze test. B: Sham-operated mice were orally administered with distilled water 60 min before sample phase trial while tacrine (2.5 mg/kg) and RJ (250 and 500 mg/kg) were administered by intraperitoneal injection and oral administration, respectively (n=10). Each data column represents the mean ± S.E.M. **P*<0.05 compared with vehicle-treated OBX group (one-way ANOVA, Dunnett's method).

3.3 RJ and tacrine improves OBX-induced non-spatial memory deficits in the novel object recognition test

In sample phase, no mice groups showed significantly differences in time spent exploring each identical object. There also no significant difference in total time spent exploring two objects between sham and OBX groups (Fig. 4 (a)). In test phase, the sham group spent a significantly longer time exploring the new object than the familiar one (P<0.05) while the vehicle-treated OBX group showed the deficit of the novel object recognition performance as shown in Fig. 4 (b). Tacrine (2.5 mg/kg, i.p.) and RJ (250 and 500 mg/kg, p.o.)-treated mice groups spent significantly longer time exploring the new object than the familiar object (P<0.01).

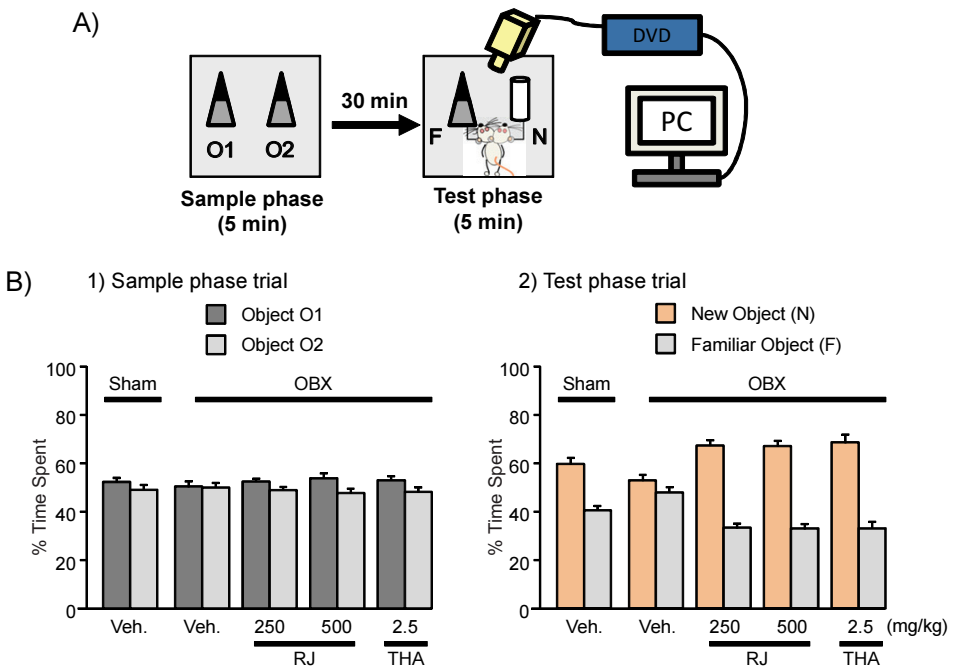

Fig. 4 Effects of tacrine and RJ on object recognition deficits in OBX mice in sample phase and test phase. A: Schematic illustration of the experimental protocol for object recognition test. B: Time each mouse spent exploring objects in the sample phase (B1) and test phase (B2) were recorded using PanLab system. Each datum represents the mean ± S.E.M. (n=10). Each data column represents the mean ± S.E.M.

3.4 RJ improved OBX-induced depression in tail suspension test

As shown in Fig. 5, the duration of immobility in TST was measured 10 days after OBX. The duration of immobility in vehicle-treated OBX mice significantly increased (25.18 ± 1.72 s) compared with that observed in sham-operated group (11.47 ± 2.13 s). One week of daily administration of imipramine (10 mg/kg, i.p.) or RJ (500 mg/kg, p.o.) significantly reduced the duration of immobility (6.48 ± 3.85 and 3.16 ± 1.94 s, respectively) while tacrine-treated group (2.5 mg/kg, i.p.) showed no reducing effect on the immobility (17.44 ± 3.75 s).

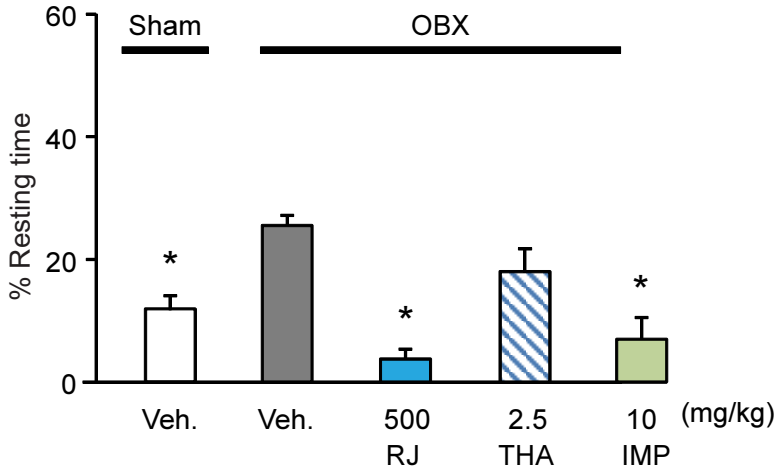

Fig. 5 Effects of tacrine (2.5 mg/kg, i.p.), imipramine (10 mg/kg, i.p.) and RJ (500 mg/kg, p.o.) on OBX-induced depressive behavior in the TST. Each data column represents the mean ± S.E.M. *P<0.05 vs. % resting time in vehicle-treated OBX mice (one-way ANOVA, Dunnett's method).

3.5 Effects of RJ on expressions of cholinergic marker genes

The expression levels of muscarinic M_1 and ChAT mRNA in the hippocampus of vehicle-treated OBX mice were significantly lower compared with sham-operated group (0.23 ± 0.04 and 0.41 ± 0.03, respectively for muscarinic M_1 mRNA and 0.12 ± 0.02 and 0.56 ± 0.28, respectively for ChAT mRNA). Nevertheless, the expression levels of M_1 and ChAT mRNA were up-regulated in OBX mice treated with tacrine (2.5 mg/kg, i.p.) or RJ (250 and 500 mg/kg, p.o.). The expression levels of M1 mRNA of tacrine and RJ (250 and 500 mg/kg, p.o.) treated groups were 0.35 ± 0.02, 0.38 ± 0.01 and 0.48 ± 0.04, respectively (Fig. 6 (A)) while the expression levels of ChAT mRNA of tacrine and RJ (250 and 500 mg/kg, p.o.) were 0.59 ± 0.11, 0.17 ± 0.01 and 0.24 ± 0.03, respectively (Fig. 6 (B)).

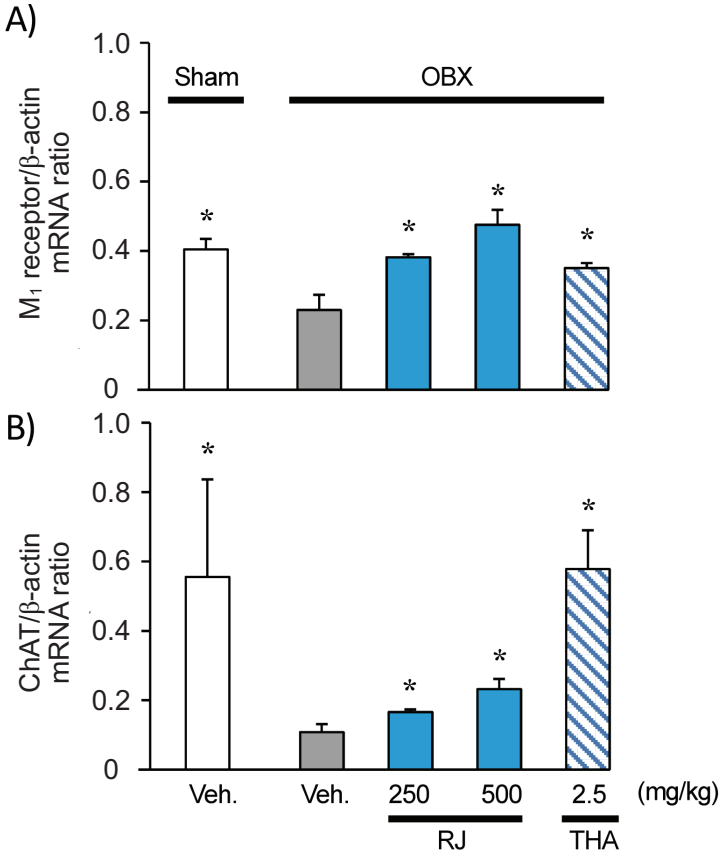

Fig. 6 Effects of tacrine and RJ on M1 (A) and ChAT (B) mRNA expression levels in hippocampus of OBX mice. Each data column represents the mean ± S.E.M. *P<0.05 vs. mRNA expression levels in vehicle-treated OBX mice (one-way ANOVA, Dunnett's method).

3.6 Effect of RJ on AChE activity

RJ (100 μg/ml) and tacrine (0.05 – 5 μg/ml) were tested for AChE inhibitory activity using Ellman's colorimetric method in 96-welled microplate. At concentration of 100 μg/ml, RJ showed low inhibitory activity (15.50 ± 4.29 %) while tacrine showed high inhibitory effect with IC_{50} less

than 50 ng/ml (Table 2). For *ex vivo* measurement of cholinesterase activity in the brain, the activity of cortical AChE in tacrine-treated groups was significantly reduced compared with the activity measured in vehicle-treated OBX mice (2.21 ± 0.21 nmol/min/mg tissue, respectively). However, the activity of cortical AChE in OBX mice treated with RJ (500 mg/kg, p.o.) was not significantly different compared with the activity of vehicle-treated OBX mice.

Table 2. *In vitro* inhibitory effects of tacrine on acetylcholinesterase activity

Concentration (g/ml)	% inhibition
0.05	77.14 ± 0.60
0.25	90.56 ± 0.25
0.5	93.67 ± 0.13
2.5	95.94 ± 0.06
5	95.76 ± 0.42

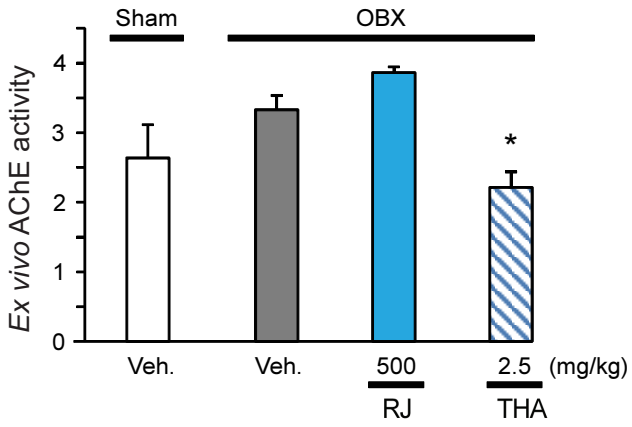

Fig. 7 Effects of RJ and tacrine on ex vivo acetylcholinesterase activity in OBX mice. *P<0.05 vs. acetylcholinesterase activity in vehicle-treated OBX mice (one-way ANOVA, Dunnett's method).

Discussion

The effects of *Thunbergia laulifolia* leaf extract (RJ) on OBX induced short term memory deficit were investigated. The results revealed that RJ treatment could ameliorate the impairments via restoring the function of the central cholinergic system down-regulated by OBX. In this study, a modified version of the Y-maze test and novel object recognition test (ORT) were employed to elucidate short-term spatial working memory and non-spatial working memory, respectively. Previous reports have shown that the modified Y-maze test and ORT are the experimental paradigms appropriate to evaluate anti-dementia activities of drugs including natural products [15, 17]. In modified Y-maze test, treatment of tacrine ameliorated spatial working memory performance indicated by the increasing of total time spent exploring new arm. However, treatment of RJ did not improve spatial working memory deficit induced by OBX. Furthermore, the effect of RJ on memory deficit using novel object recognition test was studied.

In sample phase trial, no significant difference in total time spent exploring two identical objects was observed between sham and OBX groups, indicating no differences in ability to recognize objects between animals. In test phase, the results showed that sham-operated mice spent more time exploring new object, while vehicle treated OBX mice showed no total time difference between familiar and new object indicating the impairment of non-spatial object recognition memory. Administration of RJ and tacrine could significantly ameliorate OBX induced object recognition deficit suggesting the ability of RJ in the therapy of cognitive deficits in human in the future.

The therapeutic potential of RJ for cognitive deficit and possible involvement of central cholinergic systems in its mechanisms of action were next examined. The results revealed that mice in vehicle-treated OBX group exhibited down-regulated expression levels of both ChAT and M_1 muscarinic mRNAs in hippocampus, a brain region responsible for spatial learning and memory performance indicating the deficit of cognitive performance and pathological changes in central cholinergic systems confirming the results from previous reports [12, 15]. Administration of RJ and tacrine increased the expression levels of ChAT and muscarinic M1 receptor mRNA in OBX mice However, the RJ administration did not reduced the activity of cortical AChE in OBX mice. The results suggest that RJ can protect cognitive function against degeneration of central cholinergic system caused by OBX via the reversal of expression level of ChAT and muscarinic M_1 receptor mRNA in hippocampus.

The depression model induced by bilateral olfactory bulbectomy (OBX) has been validated in rats as a good model of depression characterized by behavioural, neuroendocrine and neurochemical changes similar to human depression [18]. Moreover, the cholinergic neuronal activity does not only involve in cognitive function, but also in the depressive behavior. Therefore, the anti-depressive like effect of RJ was investigated using TST. RJ processed significantly antidepressant activity in OBX mice. However, the mechanisms of action are still unknown.

In conclusion, RJ showed improving effects on non-spatial short term memory in OBX mice which could mediate by the up regulation of the expression levels of ChAT and muscarinic M_1 receptor mRNA. Moreover, RJ also showed *in vivo* antidepressant effect in OBX mice. The results suggest the beneficial of RJ in treatment of depression and improvement cognitive impairment related to Alzheimer's disease by improving dysfunction of central cholinergic systems.

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