Effect of *Acanthopanax gracilistylus W. W. Smith* on olfactory bulbectomized – induced cognitive deficit in mice and its molecular mechanism.

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1. Abstract:

Aim: ***Acanthopanax gracilistylus W. W. Smith*** (*AG*) is commonly used as a tonic to strengthen the tendons and bones as well as to improve learning and memory in traditional medicine. However, no scientific evidence is available to support the cognitive improvement effect of AG. This study aimed to clarify the effects of alcoholic extract of AG (AG-E) on cognitive deficits in olfactory bulbectomized (OBX) mice, one of the animal models of Alzheimer's disease (AD).

Methods: OBX ddY mice were treated with AG-E at a daily dose of 180 mg and 360 mg/kg via feeding needle for 1 week before and continuously 3 days after OBX. Administration of the reference drug tacrine (2.5 mg/kg/day, i.p.) to OBX mice was started 3 days after OBX. Cognitive performances of the animals was analyzed using the novel object recognition test (ORT), modified Y maze test, fear conditioning test (FC) and tail suspension test (TST) to elucidate non-spatial short term memory, spatial working memory, long-term memory, and depression-like behavior, respectively.

Results: OBX caused impairment of non-spatial short term memory, spatial working memory, depression-like behavior and long-term memory. OBX-induced memory deficits were reversed by AG-E and tacrine.

Discussion: These findings suggest that AG-E may have a potential to improve cognitive deficits and depression-like behavior in a model of OBX but further experiments will be required to test this hypothesis.

2. Introduction

Dementia is one of the psychiatric disorders caused by aging and life style-related diseases. The most common cause of dementia in the aged people is Alzheimer's disease (AD). AD is a progressive brain disorder and results in learning and memory deficit, unusual behavior, and a

decline in thinking. The number of AD patients is increasing dramatically not only in advanced countries but also in developing countries. Therefore, it is necessary to develop drugs without/ mild side effects for prevention and therapy of AD. Medicinal herbs are the best candidate sources for development of effective AD drugs.

Some species of *Acanthopanax* such as *Acanthopanax senticosus* (Rupr. et Maxim.) Harms (*Eleutherococcus senticosus* Maxim), *Acanthopanax divaricatus*, *Acanthopanax obovatus, Acanthopanax gracilistylus W. W. Smith* (AG) have long been used in Vietnamese traditional medicine expecting tonic action, improvement of mood and cognitive deficits, immuno-modulation and anti-tumor activity [1,2]. *Acanthopanax gracilistylus W. W. Smith* (AG) is also a species of *Acanthopanax* genus that located in Ha Giang province, Vietnam. It is a valuable medicinal plant described in the Red Data Book of Vietnam for conversation. In traditional medicine, AG is commonly used as a tonic to strengthen the tendons and bones as well as to improve learning and memory. It is also effective for the cure of rheumatism and arthralgia [3]. Some lines of evidence show that AG has effects on inflammatory responses, inhibits human platelet aggregation, and exhibits antioxidant and anti-tumor activity [4, 5]. However, no scientific evidence is available to support the cognitive improvement effect of AG.

In our preliminary study where the *in vitro* acetylcholinesterase inhibitory activity (AChE) and neuroprotective effect of ethanol extract from AG were screened, we found that ethanol extract at dose of 1 mg/ml inhibited the AChE activity by 49.1%. Furthermore, this extract exhibited potential neuroprotection activity against β-amyloid$_{25\text{-}35}$-induced neuronal cell damage. Based on these results, this study evaluated the effect of AG on dementia-related behavioral performance using an animal model of OBX.

3. Materials and Methods

3.1. Plant materials and preparation of crude extract:

AG was collected from Ha Giang Province (Northern mountain of Vietnam). It was identified by Dr. Pham Thanh Huyen, Department of Medicinal Plant Resources, National Institute of Medicinal Materials (NIMM, Vietnam). The sterm-bark of AG was dried, grinded into semi-powder, and hot extract with 70% ethanol. Ethanol extraction was condensed under reduced pressure. The concentrated extract was then dissolved in water for 24 hrs and rejected residue by vacuum-filter. Evaporate water under reduced pressure and dry under vacuum to get the powder.

3.2. Animal

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 (light on: 07:30-19:30) at 22 ± 1°C. 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 17:00. The present studies were conducted in accordance with the Guiding Principles for the Care and Use of Animals and were approved by the Institutional Animal Use and Care

Committee in the University of Toyama. The animals were divided to the following 5 groups:

1. Group S: Sham (S).
2. Group OBX: OBX (O).
3. Group A: OBX + 0.18 g ethanol 70 % extract of *Acanthopanax gracilistylus* sterm-bark (A).
4. Group B: OBX + 0.36 g ethanol 70 % extract of *Acanthopanax gracilistylus* sterm-bark (B).
5. Group T: OBX + 2.5 mg/kg (i.p.) tacrine

3.3. Surgical operation:

OBX of mice was conducted as previously reported (6, 7). Briefly, the mice were anesthetized with sodium pentobarbital then fixed on stereotactic instrument (Narishige, Tokyo, Japan). The skull covering the bulb was exposed by skin incision; 1 mm 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 the behavioral tests, all the animals were sacrificed and the operated lesion was verified visually. The data from animal with less than 70 % removal or with no intact cortex were excluded from the analysis. Sham operation was performed in a similar way without remove of the bulb. At the end of experiments, the olfactory bulbs of sham group mice were confirmed to be intact.

3.4. Drug administration:

Drug treatments and experiment schedule were done according to the schematic in the figure 1.

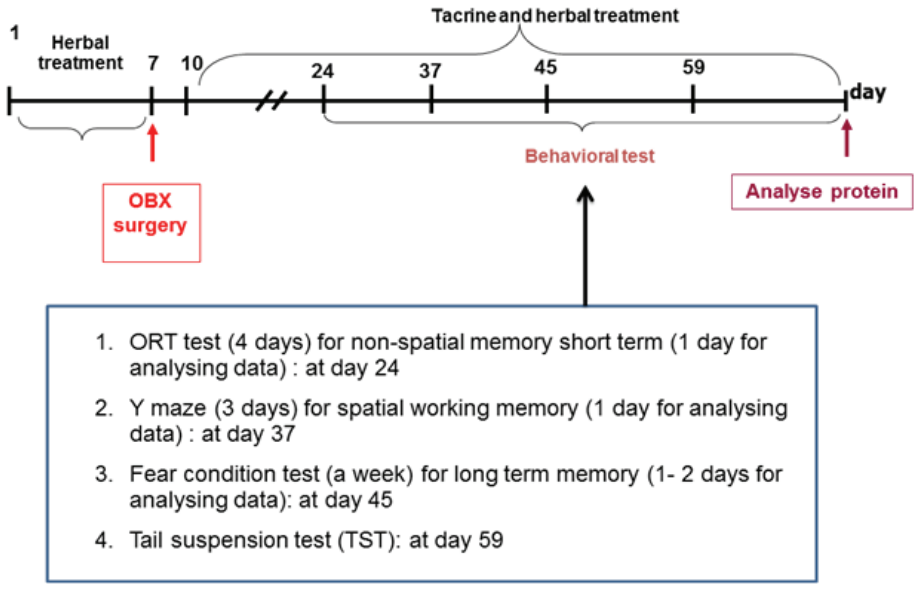

Fig. 1 Schematic drawing of experimental schedule

3.5. Behavioral study

Behavioral experiments were usually conducted during a period from 09:00 to 17:00 according to the previous reports from Dr. Matsumoto's laboratory, Institute of Natural Medicine, University of Toyama.

3.5.1. Object recognition test (ORT) (6,8).

ORT was conduct 2 weeks after operation days.

ORT consisted of a sample trial and a test trial. Before starting experiment, mice were habituated with the experimental room for at least 30 – 60 min. In the sample trial, object 1 and 2 are exactly the same. In the test trial, object 1 or 2 was replaced by object 3 which had a shape differing from object 1. SMART system® (PanLab, Barcelona, Spain) was used to analyze total time the animals spent to explore each object in the sample and test trials. After each trial, the chamber was cleaned with 70 % ethanol.

3.5.2. Modified Y maze test (6).

Y-maze test was conducted 2 weeks after ORT. The test consisted of a sample trial and a test trial. Before starting experiment, mice were habituated with the experimental room for at least 30 – 60 min. Three different cues were set above the middle of each arm. In the sample trial, each mouse was individually placed in the maze with one of 3 arms closed, and allowed to explore the arms freely for 5 min. In the test trial, each mouse was again placed in the maze with all 3 arms opened, and allowed to explore the arms freely for 5 min. SMART system® was used to analyze the total time that mice explore each arm (only in the test trial). The chamber was clean with 70 % ethanol and dried after each trial.

3.5.3. Fear conditioning test (FCT) (6,9).

FCT was conducted 1 week after the modified Y-maze test. This test consisted of a training session (first day), contextual memory test (1 day after the training session) and an auditory memory test (5 days after training session). Before starting experiment, mice were habituated with the experimental room for at least 30 – 60 min. The chamber for fear conditioning consisted of a transparent acrylic chamber and a stainless-steel grid floor equipped with an electric shock generator/scrambler, SGS-002R, CS Controller CSS-001R,

and Cycle TimerCMTR (Muromachi Kikai, Co. Ltd., Tokyo, Japan). The apparatus was placed in a sound proof observation box (MC-050/CM, Muromachi Kikai, Co. Ltd., Tokyo, Japan) through which the auditory tone (2.9 kHz, 80 dB) (SonalertR, Mallory Sonalert Products Inc., Indianapolis, IN, USA) was delivered to the animal. The chamber was cleaned with 70 % ethanol after each session. Animal behavior in the chamber was video-recorded for later analysis.

In the training session, the animals were individually placed in the fear conditioning chamber and allowed to explore freely for 3 min for habituation and then they received an acoustic tone and foot shock (0.8 mA, 2s) at a 1-min interval. The contextual memory test and auditory-dependent fear memory test were conducted one and 5 days after the training, respectively. In the contextual fear memory test, the animal was placed in the same chamber to provide the contextual stimuli and allowed to move freely for 6 min without tone or foot shock. One min after placing the animal in the chamber, freezing behavior during the 5-min period was recorded. In the auditory test, the animal was placed in a chamber different from that used in the training

session for 4 min and then received the tone for 2 min to record the freezing behavior. The animal behavior was video-recorded and analyzed using the SMART system.

3.5.4. Tail suspension test (TST).

TST was used to assess the anti-depressant-like effect of AG extract. The test was conducted 1 week after FCT. The test was conducted as previously reported (Mizuki et al., 2014a,b). Before starting experiment, the animals were habituated in the experimental room for at least 30 – 60 min. Sixty min after the administration of test drugs, each mouse was individually suspended 50cm above the floor by the tail with adhesive tape placed approximately 2 cm from the tip of the tail. Immobility was defined as a state with movement speed of no more than 0.05cm^2/s using the SMART system and was recorded for 8min. The performance during the last 6-min period was analyzed.

3.7. Data analysis

The data are expressed as the mean ± SE. The data from the behavioral tests and neurochemical experiments were analyzed by paired or unpaired Student's t-test or one-way analysis of variance (ANOVA) follow by a post hoc test (Dunett'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., Richmon, CA, USA)

4. Results

4.1. AG improves OBX-induced non- spatial memory deficit in the novel object recognition test

Using the novel object recognition test, we elucidated the effect of AG on the non-spatial cognitive performance of OBX mice (Fig. 2). In the sample trial, the times spent exploring each identical object (O1 and O2) were not different in each group. However, in the test trial, the sham and AG groups (A and B group) spent a significantly longer time exploring the novel object (O3) than exploring the familiar one (O1). In this case, tacrine (2.5 mg/kg)-treated OBX mice spent time exploring the familiar object as long as exploring the novel one. This result differed from the results reported by others including this laboratory.

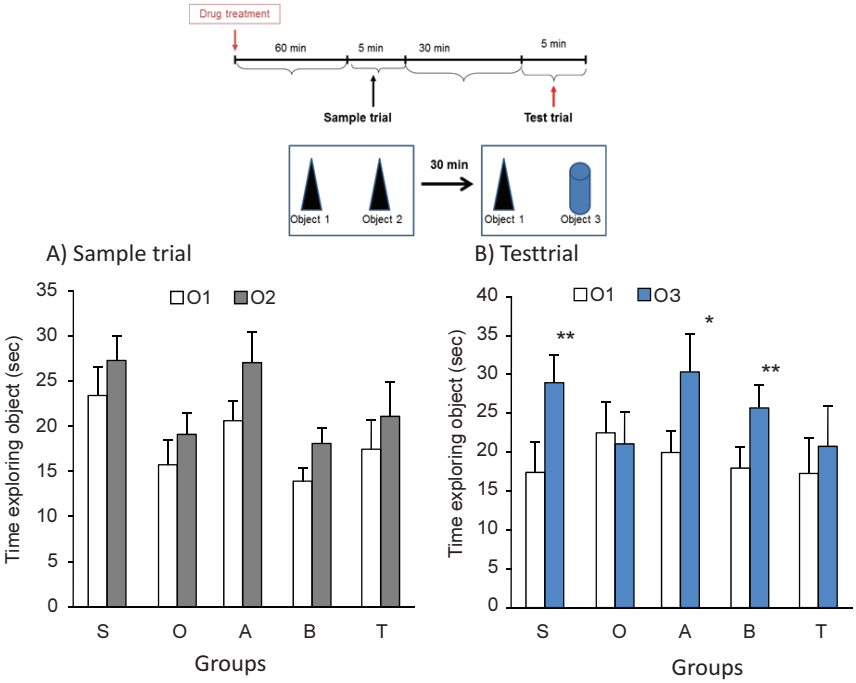

Fig. 2 Effects of AG on OBX-induced short-term memory deficits in the object recognition test. Each data column represents the mean ± S.E.M (n=8-9). *P < 0.05 and **P<0.01 vs. time spent exploring a familiar object (pair t-test).

4.2. AG had the trend on improvement OBX-induced spatial working memory deficits in the modified Y maze test.

Using the modified Y maze test, we evaluate the effect of AG on spatial working memory on OBX mice. The results showed on the figure 3.

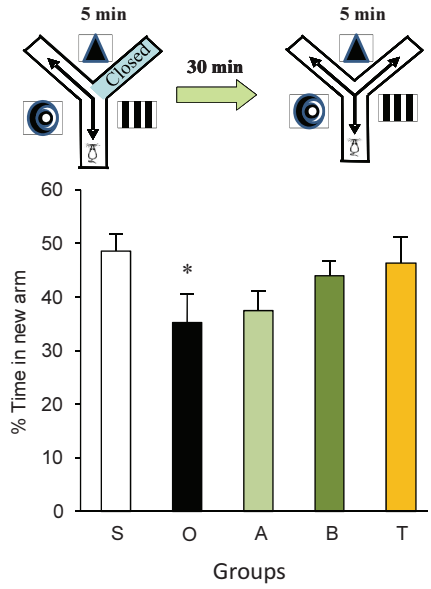

Fig. 3 Effect of AG on OBX-induced spatial working memory deficit in the modified Y maze test. Each data column represents the mean ± S.E.M (n=9). * P < 0.05 vs. S group (t-test).

In the Fig. 3, total time that the mice in the sham group used in the novel arm are longer than that of OBX group. The higher dose of AG (B group) and tacrine group have time in novel arm are no longer than that of OBX group, though they has the trend on improvement OBX-induced spatial working memory deficits in the modified Y maze test. The result of tacrine group was not the same as theory and others at this laboratory maybe because of quality of tacrine (tacrine vial was used for long time with many times taken out of low temperature (4 ℃), so that in the ORT tacrine had no any effect even the trend. In the Y-maze test, new tacrine was used but only 4 days before the experiment so the result was still not good but it had the trend on increasing the time spending in the novel arm.

4.3. BM ameliorated OBX induced long term memory deficit in the fear conditioning test

To determine the effects of AG on long-term memory deficits induced by OBX, we used fear conditioning test (FC). One week after finishing the modified Y maze test, the fear conditioning test was performed. Mice were fear-conditioned to the context and auditory stimuli by electrical foot shocks as unconditioned stimuli, 1 day and 5 days after, freezing responses to the contextual and auditory stimuli were recorded, respectively. The results showed in the figure 4.

As shown in Fig. 4, AG at a higher dose (group B) and tacrine increased freezing time in the contextual memory test compare to that of the group O indicating that AG and tacrine improved hippocampus-dependent memory formation. In the auditory memory test, only tacrine increased the freezing time compared to the group O.

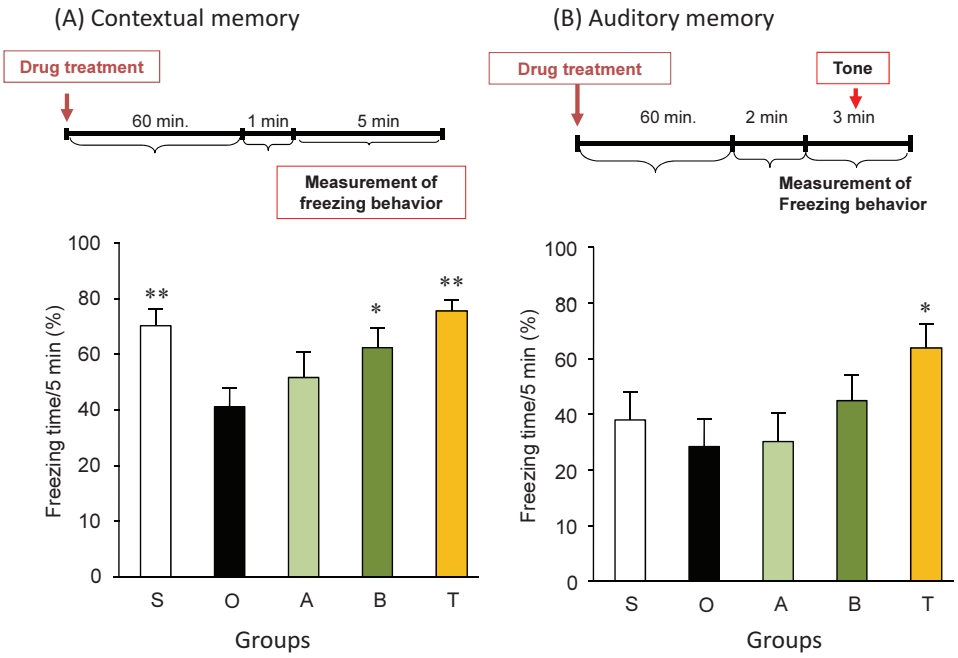

Fig. 4 Effect of AG on OBX-induced long-term memory deficit in the fear conditioning test. Each data column represents the mean ± S.E.M (n=9). (A) Contextual memory performance; (B) Auditory memory performance were analyzed as described in the text. * $P < 0.05$ and **$P < 0.01$ vs. O group (ANOVA followed by post hoc Student-Newman-Keuls test).

4.4. Effect of AG on depression-like behavior of OBX mice in the TST.

To evaluate the effect of AG on depression-like behavior of OBX mice, we used tail suspension test (TST). In this experiment, the group O showed a significantly longer immobility time than the group S (ANOVA followed by post hoc Student-Newman-Keuls test). Tacrine had no effect on depression-like behavior of OBX mice. In fact, the immobility time of the group T was the same as that of the group O. The group B tended to reduce immobility time compare with the group O (Fig. 5).

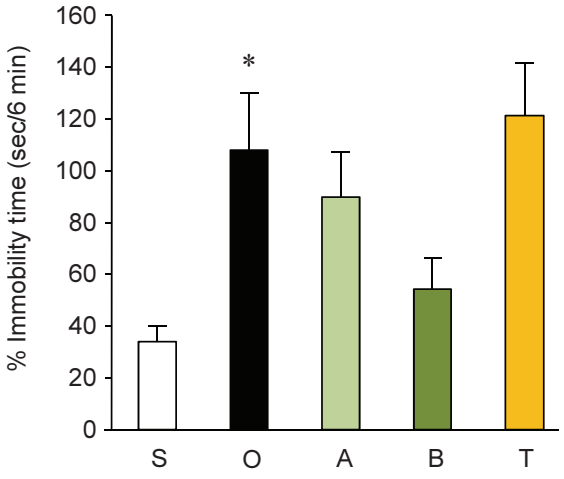

Fig 5 Effect of AG on OBX-induced depression-like behavior in the tail suspension test. Each data column represents the mean ± S.E.M (n=9). * $P < 0.05$ vs. S group (ANOVA followed by post hoc Student-Newman-Keuls test).

5. Discussion

In this study, we investigated the effects of AG on OBX-induced cognitive deficits and depression-like behavior. We first elucidated the effect of AD on short-term non-spatial working memory using the object recognition test. In the sample trial of ORT, no significant difference in total time spent exploring two identical objects was observed in all groups, indicating that OBX does not change vision and/ or an ability to visually recognize

objects. In the test trial, sham-operated mice spent more time exploring a novel object, while vehicle-treated OBX mice failed to discriminate between familiar and novel object. AG treatment significantly ameliorated OBX-induced deficit of novel object recognition. The administration of AG and tacrine could attenuate OBX-induced deficits of long-term memory in the fear conditioning test, especially in the contextual test. Though in the Y-maze test, AG only had trend on exhibited impaired spatial working memory performance and in the TST, AG group (Group B) had reduced immobility time induced by OBX. These findings suggest that AG-E may have a potential to improve cognitive deficits and depression-like behavior in a model of OBX but further experiments will be required to test this hypothesis.

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