

Original Article

Effects of *Acori Graminei Rhizoma* on Scopolamine-induced Amnesia in Rats

Bo-Kyoung Park, Sang-Yeon Min, Jang-Hyun Kim

Department of Pediatrics, College of Oriental Medicine, Dongguk University

Objectives : Amnesia is the loss or impairment of memory, caused by physical injury, disease, drugs, or emotional trauma. Recently, the average life span is increasing, while at the same time, the incidence of dementia-like diseases in conjunction with amnesia are also increasing. Therefore learning and memory are very important issues in modern society. Ancient Korean physicians used several herbs to treat dementia and these herbal effects were described in Korean herbal books. Among them are some reports on several cognitive-enhancing herbs which have since been shown to improve dementia in recent pharmacological studies, such as Panax ginseng; however, the facilitatory effects of many Korean cognitive-enhancing herbs on learning and memory are limited. Learning and memory are essential requirements for every living organism in order to cope with environmental demands; cholinergic systems are known to be involved in learning and memory.

Methods : In this study, the effects of *Acori graminei rhizoma* (AGR, 石菖蒲) on learning and memory were investigated by Morris water maze, eight-arm radial maze, and the effects on the central cholinergic system of rats injected with scopolamine.

Results : In the water maze, the experimental animals were trained to find a platform in a fixed position for 6 days and then received a 60 sec probe trial in which the platform was removed from the pool on the 7th day. In the eight-arm radial maze, the animals were tested four times per day for 6 days. Scopolamine impaired performance of the maze tests and reduced activity of acetylcholinesterase (AChE) in the hippocampus, which is a marker for the central cholinergic system. There were significant reversals from the scopolamine-induced deficits on learning and memory in these tests, through daily administrations of AGR (100 mg/kg, p.o.) over 14 consecutive days. These treatments also reduced the loss of cholinergic activity in the hippocampus induced by scopolamine.

Conclusions : These results demonstrated that AGR ameliorated learning and memory deficits by affecting the central acetylcholine system.

Key Words : *Acori Graminei Rhizoma*, amnesia, acetylcholinesterase (AChE)

Introduction

As a result of the high increase in information and competitiveness in today's society, people tend to suffer from stress-related symptoms as well as difficulties with memorization. These difficulties in memorization tend to be related to aging of the brain^{1,2)}. These problems have become large issues

over recent years, and a matter of concern in learning ability and memorization among men's highly qualified mental activities³⁾.

Learning promotes continual changes in the brain's functioning through experience. Memory means the ability of using rapid recall in the conscious state in a timely manner. Learning and memory are in the undividable relation, and the learning can be meas-

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• Correspondence to : Jang-Hyun Kim

Oriental Medicine Hospital, Dongguk University 1090-1, Sukjang-dong, Gyeongju, Gyeongbuk, Korea,
Institute of Oriental Medicine Dongguk University

Tel : +82-54-770-1260, Fax : +82-54-770-2281, E-mail : kjh@dongguk.ac.kr

ured by the quantity and the quality of the memory.

In oriental medical theories, conceptions of memory and learning are included in the functions of the heart(Xin, 心), the mind(Shen, 神), the essence(Jing, 精), the energy (Qi, 氣), and the brain(Nao, 腦).

The heart controls the functions of spirit and vital activity. The mind is considered as conception of vital phenomenon and thinking consciousness, the essence as conception of vital original matter, the energy as conception of the vital activity. These all are expressed by the mind externally^{4,5}. The brain is the place where the essence congeries into the mind, and the mind converted from the brain emerges in the whole body, the sensory organs and carries vital function⁶.

Acori Graminei Rhizoma(AGR) is a herb used frequently in oriental medicine. Clinically, the effects of AGR are known as awakening one's consciousness, removal of sputum, and relieving autism. Experimentally, AGR increases cerebral blood flow depending on the concentration⁷ causes diverse responses of blood pressure and regional cerebral blood flow⁸) and possesses strong inhibitory effects of apoptosis in the nervous system and effects of neuronal regeneration against the apoptosis of neuroblastoma cells by CT105 expression⁹.

Therefore, AGR has been considered to improve learning and memory, and to regulate physiological activities by neuroprotective effects and improvement of cerebral blood flow. This paper describes the effects of AGR extract on the deficits of learning and memory induced by scopolamine in rats; by using a water maze task, an eight-arm radial maze task, and AchE immunohistochemistry.

Materials and Methods

1. Animals

Adult male Sprague-Dawley rats weighing 270 ± 10 g were obtained from Samtako (Gyeonggi-do, Korea). All animals were housed in groups of five or six with continuous access to food and water *ad*

libitum and were maintained on a 12 hours light/dark cycle regulated at 23°C. The experiments began at least 7 days after their arrival in individual home cage.

2. Preparation of methanol extract of *Acori Graminei Rhizoma* (AGR)

100 g of *Acori Graminei Rhizoma* (AGR, Jungdo Inc. Seoul, Korea) was cut into small pieces and extracted three times in a reflux condenser for 24 hours each with 85% methanol. The solution was combined, filtered through Whatman No.1 filter paper, and concentrated using a rotary vacuum evaporator followed by lyophilization. The yields of AGR were about 14.0% (w/w).

3. Procedure and administration of AGR

Experimental animals were divided into 3 groups; Sham as a normal group comprised 9 rats. Saline + scopolamine as a control group and AGR + scopolamine as the experimental group each comprised 10 rats.

The general procedures for scopolamine injection were the same for each group, except that saline (i.p.) was injected into the sham group, whereas scopolamine (Sigma, St. Louis, MO) was injected into rats in the control group at a concentration of 2 mg/kg of saline.

AGR was dissolved in saline (100 mg/ml). A suspension of AGR (AGR + scopolamine group, 100 mg/kg per day) or saline (sham group) was administered intraperitoneally for 2 weeks. During scopolamine treatment, the saline + scopolamine and AGR + scopolamine groups received scopolamine (2 mg/kg for 1 week) and 30 min later the Morris water maze started and the eight-arm radial maze was started for one week.

4. Morris water maze task

The water maze was a circular pool (painted white, 2.0 m in diameter, 0.35 m high) constructed from fiberglass. The pool contained water that was

maintained at a temperature of 22 ± 2 °C. The water was made opaque by the addition of 1 kg of powered milk. During testing in the water maze, a platform, 15 cm in diameter, was located 1.5 cm below the water in the one of four locations in the pool, approximately, 50 cm from the side walls. The pool was surrounded by many clues external to the maze. A video-camera was mounted to the ceiling above the pool and was connected to a video-recorder and tracking device (S-MART, Pan-Lab, Spain), which permitted on- and off-line automated tracking of the path taken by the rat. The animals received four trials per session. The rats were trained to locate the hidden escape platform, which remained in a fixed location throughout testing. Trials lasted a maximum of 180 sec and the latency to find the submerged platform was recorded. The animals were tested in this way for 6 days, and then they received a probe trial on the 7th day. For the probe trial, the platform was removed from the pool and the animal released from quadrant opposite to where the platform would have been located. The length of the trial was 60 sec, after which the rat was removed from the pool. The time the rat spent searching for the previous location of the platform, was recorded, and used as a measure of retention.

5. Eight-arm radial maze task

The maze consisted of a central platform 38 cm in diameter, with eight arms extending radially. Each arm was 70 cm in length and 10 cm in width with wooden sidewalls 25 cm in height (Fig. 1). Food cups for reinforcement were located in a room containing many extra-maze visual cues. For behavioral analysis, an image motion analysis program (S-MART, Pan-Lab, Spain) was used to quantify the task performance of rats in the eight-arm radial maze task. The trial continued until the test animals entered all eight arms or 5 min had elapsed. The task was performed four trials per day for 6 days. The performance of the test in each trial was assessed by three parameters: the number of correct choices in the initial eight

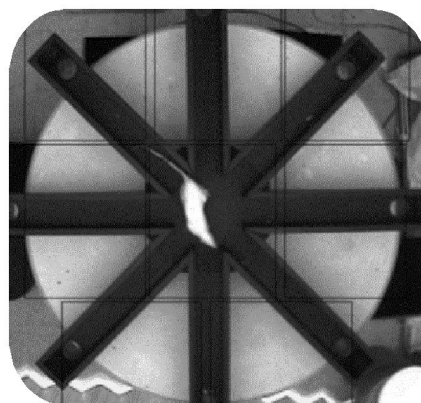


Fig. 1. Eight-arm radial maze

chosen arms, the number of errors which was defined as choosing arms that had already been visited, and the running time elapsed before the animal ate all eight pellets.

6. Acetylcholinesterase (AChE) Immunohistochemistry

At the end of the behavioral observation, the rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and then perfused through the ascending aorta with normal saline (0.9%), followed by 900 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, postfixed overnight and cryoprotected in 20% sucrose with PBS. The brains were cut by cryostat as 30 μ m coronal sections, that were processed immunohistochemically as free-floating sections. The sections were washed in PBS and incubated in the solution with 25 mg acetylthiocholine iodine for 1 hour. The solution was composed of 0.1 M sodium hydrogen phosphate buffer ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, pH 6.0) 32.5 ml, 0.1 M sodium citrate 2.5 ml, 30 mM copper sulfate 5 ml, 5 mM potassium ferricyanide 5 ml, and distilled water 5 ml. The color of mixing solution was green. The density of stained nuclei of hippocampal cells was measured using Scion image program (Scion Corp., MD).

7. Statistical analysis

The data was expressed as a mean \pm S.E.M. Group differences in the escape latency in the maze training tasks were analyzed using two-way analysis of variance (ANOVA) with repeated measures. One-way ANOVA followed by the Tukey's post-hoc test multiple group comparison was used to analyze group differences of the data collected during successive training day, probe trials, immunohistochemical assay and image analysis. A difference between groups was considered as statistically significant if the associated probability (P-value) was below 0.05.

SPSS 10.0 for Windows was used for all statistical analysis in this study.

Results

1. Morris water maze task

The results of acquisition of the Morris water maze task are depicted in Fig. 2. The escape latency differed between the groups when averaged over all sessions ($P<0.01$). Post-hoc comparisons revealed that the saline + scopolamine group needed more time to locate the platform than the AGR + scopolamine group did. During the experiment the

latency to escape diminished ($P<0.001$), but there was no relationship between the groups and the days ($P>0.15$). Post-hoc test revealed that the AGR + scopolamine group ($P<0.05$ on days 2 and 4, $P<0.01$ on day³) significantly reduced the latency of swimming time, compared with those of the saline + scopolamine group.

Analysis of the performance on the probe trial comparing the percentage of time spent swimming in the platform is illustrated in Fig. 3. The time spent around the platform among groups differed ($P<0.05$). The sham and AGR + scopolamine groups spent more time around the platform than the saline + scopolamine group ($P<0.05$ for both groups). Scopolamine severely impaired spatial cognition in the water maze task; the AGR treatment group attenuated scopolamine-induced learning and memory loss in the water maze.

2. Eight-arm radial maze task

The saline + scopolamine group showed a slower acquisition curve than the sham group as shown in Fig. 4. The saline + scopolamine group showed a greater number of errors over testing, as compared with the sham group. The AGR + scopolamine group significantly reduced the number of errors,

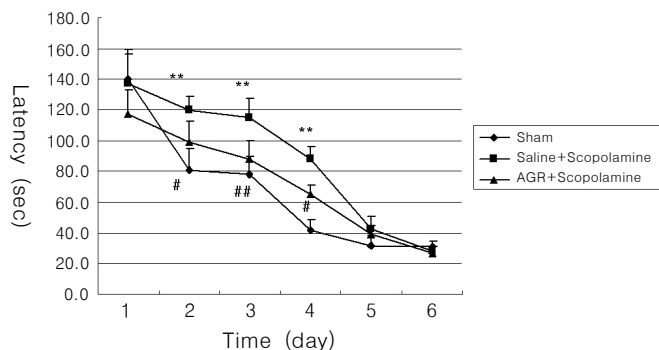


Fig. 2. Comparison of acquisition performance on the Morris water maze task among the three groups of rats. Mean swimming time traveled per trial. Mean values of the four trials per day for 6 days for each of the three groups are shown. Repeated measures of ANOVA of swimming time among the groups were performed and followed by Tukey's test.

* $P<0.05$, ** $P<0.01$ as compared with the corresponding data of the saline + scopolamine group.

$P<0.05$, ## $P<0.01$ as compared with the corresponding data of the AGR + scopolamine group.

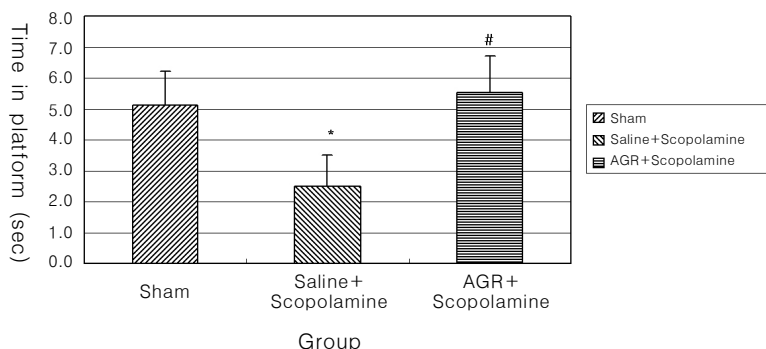


Fig. 3. Comparison of retention performance on the acetylcholic Morris water maze task among the three groups of rats. Mean percentage time of platform spent swimming per trial. Mean values of the four trials for each group are shown. Repeated measures of ANOVA of swimming time among the groups were performed and followed by Tukey's test.

* P<0.05 as compared with the corresponding data of the saline + scopolamine group.
 # P<0.05, as compared with the corresponding data of the AGR + scopolamine group.

compared with the saline + scopolamine group as seen in Fig. 4. An ANOVA (4×6) performed on the number of errors indicated a significant difference among the groups (P<0.001), a significant effect of day (P<0.001), but not a group-day relationship (P=0.451). Post-hoc analysis revealed that injections of AGR produced a progressive decrease in number of error responses, compared with that of the saline + scopolamine group. These recovering effects of

AGR were significant after 5 days (P<0.01), as shown in Fig. 4. However, there were no significant differences among the three groups in running time (P>0.12) as seen in Fig. 5. Performance in the eight-arm radial maze task was impaired in the saline + scopolamine group, and AGR treatment attenuated scopolamine-induced learning and memory damage in the eight-arm radial maze.

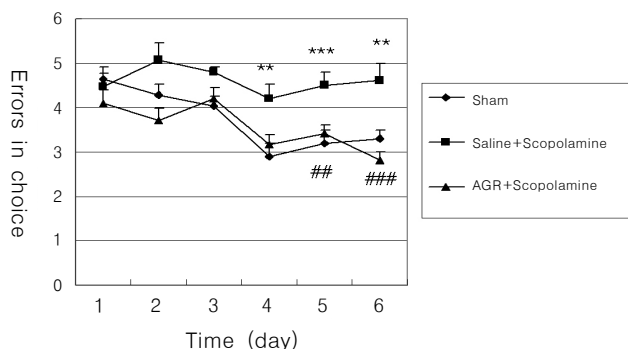


Fig. 4. Acquisition performance on eight-arm radial maze task among the three groups of rats (errors in choice). The task was started after scopolamine treatment and was performed with 4 trials per day for 6 days. Repeated measures of ANOVA of swimming time among the groups were performed, followed by Tukey's test.

** P<0.01, *** P<0.001 as compared with the corresponding data of the saline + scopolamine group.
 ## P<0.01, ### P<0.001, as compared with the corresponding data of the AGR + scopolamine group.

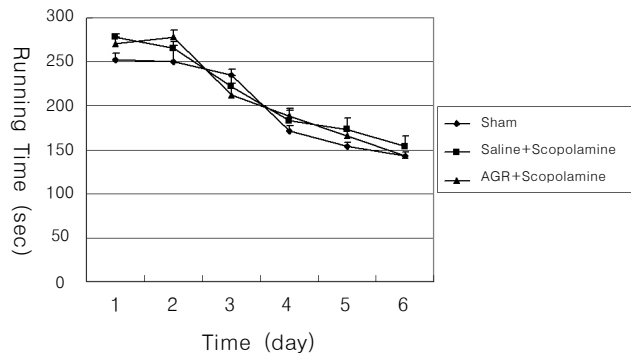


Fig. 5. Performance on eight-arm radial maze task among the three groups of rats (running time).

3. Acetylcholinesterase (AChE) Immunohistochemistry

As seen in Fig. 6 and 7, the density of AChE fibers in the hippocampal formation decreased in the saline + scopolamine group, compared to the sham group. The density of AChE neurons in the CA1 area was 100.0 ± 4.5 in the sham group, 81.0 ± 2.5 in the saline + scopolamine group, and 91.0 ± 2.5 in the AGR + scopolamine group ($P < 0.05$). Post-hoc comparisons revealed that the saline + scopolamine group was significantly different in the CA1 areas compared to the sham group ($P < 0.05$).

Discussion

Learning means the continuous change in the brain mechanically by practices or experiences, and means the stage to adapt to the environment and to take an action by using acquired information²⁾. Memory means the ability of using information in the conscious state at the time of the necessity of the result. Learning and memory are in an indivisible relationship, learning can be measured by the quantity and the quality of the memory, and learning is described as a change of behavior which is reflected by memory¹⁰⁾.

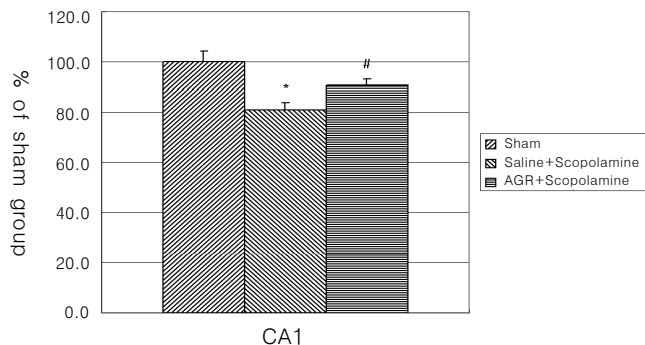


Fig. 6. The percentage (\pm S.E.M) of sham values of density of AChE stained nuclei in CA1 area of the hippocampus of the experimental groups after water maze learning task 7 days post-operatively. The AChE results were analyzed by performing separate one-way ANOVA of neurons among the groups followed by Tukey's test.

* $P < 0.05$ as compared with the corresponding data of the saline + scopolamine group.

$P < 0.05$ as compared with the corresponding data of the AGR + scopolamine group.

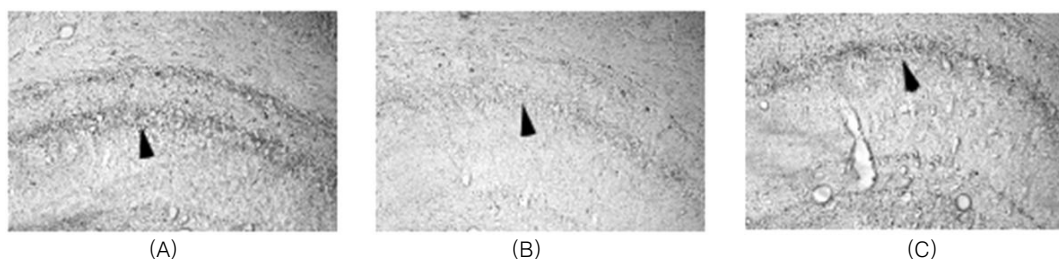


Fig. 7. Photographs showing the distribution of AchE reactive cells in the hippocampus of sham (A), saline + scopolamine (B), and AGR + scopolamine group (C) rats. Sections were cut at 30 μ m. AchE-reactive cell density can be seen in CA1 area of the hippocampus (arrows).

The brain structures, such as the hippocampus, the amygdala, and the medial septum, take a part in learning and memory¹¹⁻¹³. The medial septal nucleus and the hippocampus make the septohippocampal system. This system plays an important role in learning and memory of this kind of rodent¹⁴⁻¹⁵.

The body of the hippocampus is subdivided into CA1, CA2, CA3, and CA4 areas. It is known that the CA1 and CA3 areas have an intimate relation with learning and memory. Especially, the CA1 pyramidal neurons in the hippocampus are selectively vulnerable to transient forebrain ischemic insult¹⁶⁻¹⁷, and it is known that alcohol-induced memory disorder also has an intimate relation with deficit of CA1 pyramidal neurons¹⁸. Such selective damage of CA1 area of the hippocampus causes deficiency in learning and memory. Acetylcholine (ACh) is distributed at the subcortical area. ACh has a stimulative effect on the brain and promotes learning and memory¹⁹. Acetylcholinesterase (AChE) divides acetylcholine into acetate and choline; therefore, the measurement of amount of AChE predicts the amount of ACh²⁰.

Recently, many studies have reported about learning and memory by oriental medicine. It was reported by using Morris water maze that *Cervi Cornu Parvum* (鹿茸), *Bo Ah-tang* plus *Cervi Cornu Parvum* (補兒湯加鹿茸), *Coptis Japonica Makino* (日黃蓮), *Phellodendron Amurense* (黃柏), *Ansincheon-nyoytang* (安神清腦湯) increased the ability of memory²¹⁻²⁵, and that *Hyangbujapalmutang* (香附子八物

湯) increase the ability of memory by using radial-arm maze²⁶.

It was reported that *Jowiseungchungtang* (調胃升清湯), *Hyongbangjiwhangtang* (荊防地黃湯) and *Sahyangsohapwon* (麝香蘇合元) improved the ability of learning and memory in mice had brain damage which is similar to Alzheimer's disease²⁷⁻²⁹. Many other studies have researched learning and memory by using the Morris water maze and radial-arm maze.

The Morris water maze is a major trial method to evaluate learning and memory about the environmental space, and it can also investigate the long-term memory of the test animals by using only environmental space-information without olfactory information or any other information. The Morris water maze can measure the ability of environmental space-information-based memory^{14,30}. The radial arm maze was designed by Olton³¹. It gives the test animals only environmental space information, which is similar to the Morris water maze. It can measure the working memory of the test animals, which is a type of short term memory³²⁻³⁴.

In this study, *Acori Graminei Rhizoma* (AGR) is evaluated the effects on learning and memory. AGR is a herb used frequently in oriental medicine. Clinically, the effects of AGR are known as awakening one's consciousness, removal of sputum, tranquility, and so on⁷. Experimentally, AGR increases cerebral blood flow depending on concentration⁷, causes diverse responses of blood pressure and regional cerebral blood flow⁸, and possesses

strong inhibitory effects of apoptosis in the nervous system and repairing effects against the degeneration of neuroblastoma cells by CT105 expression⁹⁾.

Therefore, AGR has been considered to improve learning and memory, and to regulate physiological activities. This paper describes the effect of AGR extract on deficiency in learning and memory induced by scopolamine in rats, by using water maze task, eight-arm radial maze task, and AchE immunohistochemistry.

This research was an observation of rats injected with scopolamine, which decreases the acetylcholinergic nervous activity, which observed the rat's ability of learning and memory by using the Morris water maze and eight-arm radial maze. After that, the rats were anesthetized, their brains were extracted, and the inhibiting level of AchE in the hippocampus, which secretes Ach in medial septum, was measured to analyze the effects on learning and memory.

In the acquisition test of the Morris water maze task, the AGR + scopolamine group took less time than the sham and saline + scopolamine groups in the swimming time of the probe trial by Tukey's post-hoc test. During the experimental period, the swimming time decreased ($P < 0.001$), but there was no relationship between each group and each day ($P > 0.15$). By Tukey's post-hoc test, the AGR + scopolamine group took significantly less time than the other groups on the 2nd, 3rd, and 4th days ($P < 0.05$ on day 2 and 4, $P < 0.01$ on day³).

In the retention test of the Morris water maze task, each group was different with each other in the swimming time spent around the platform ($P < 0.05$). The swimming time in the sham and AGR + scopolamine groups was longer than in the saline + scopolamine group ($P < 0.05$ for each).

In eight-arm radial maze task, the saline + scopolamine group had lower acquisition and more errors than the sham and AGR + scopolamine groups. Each group was different with each other by ANOVA analysis ($P < 0.001$). Among days, there were significant differences ($P < 0.001$), but in the relationship between each group and each day, there was no

significant difference ($P = 0.451$).

By Tukey's post-hoc test, AGR decreased the number of errors. The recovering effects of AGR showed no significance at 5th day and later (Fig. 3). However, for the running time, there was no significant difference among the groups ($P > 0.12$).

In AchE immunohistochemistry, density of AchE neurons was measured at the CA1 area of the hippocampus. This density decreased in the saline + scopolamine group, compared with the sham and AGR + scopolamine groups. In the CA1 area, the density of AchE neurons was 100.0 ± 4.5 in the sham group, 81.0 ± 2.5 in the saline + scopolamine group, and 91.0 ± 2.5 in the AGR + scopolamine group ($P < 0.05$). By Tukey's post-hoc test, the saline + scopolamine group was significantly different from the sham group in the CA1 area ($P < 0.05$).

These results suggest that AGR has effects to increase the ability of learning and memory, because it could reinforce the Ach's actions through inhibiting the AchE's actions induced by scopolamine.

Conclusion

In order to research the effects of *Acori Graminei Rhizoma*(石菖蒲) extract on the deficiency of learning and memory induced by scopolamine in rats, acquisition performance and retention performance were investigated by using water maze task and eight-arm radial maze task; the density of acetylcholinesterase at CA1 area of the hippocampus was measured by acetylcholinesterase immunohistochemistry.

1. In the acquisition test of the Morris water maze task, the AGR + scopolamine group took significantly less time than the other groups on the 2nd, 3rd, and 4th days.
2. In the retention test of the Morris water maze task, the swimming time in the sham and AGR + scopolamine groups was significantly longer than in the saline + scopolamine group.
3. In eight-arm radial maze task, the saline + scopolamine group had significantly lower

acquisition and more errors than the sham and AGR + scopolamine groups. The AGR + scopolamine group had fewer errors than the saline + scopolamine group.

4. In acetylcholinesterase immunohistochemistry, the density of acetylcholinesterase neurons at the CA1 area of the hippocampus significantly decreased in the saline + scopolamine group, compared with the sham and AGR + scopolamine groups.

These results demonstrated that *Acori Graminei Rhizoma*(石菖蒲) ameliorated deficits of learning and memory by acting on the central acetylcholine system. This study suggests evidence of *Acori Graminei Rhizoma* as a treatment for Alzheimer's disease, mental retardation, and learning disorders.

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