Effects of Yukmijihwang-tang Derivatives (YMJd) on Ibotenic Acid-Induced Amnesia in the Rat

Moonkyu Kang, Ji-Hyun Kim, Chongwoon Cho, Kwang-Yeun Lee, Minkyu Shin, Moochang Hong, Insop Shim, and Hyunsu Bae

The present study investigates the effects of Yukmijihwang-tang Derivatives (YMJd) on learning and memory through the Morris water maze task and the central cholinergic system of rats with excitotoxic medial septum (MS) lesion. In the water maze test, the animals were trained to find a platform in a fixed position for 6 d and then received a 60-s probe trial in which the platform was removed from the pool on the 7th day. Ibotenic lesion of the MS showed the impaired performance in the Morris water maze test and severe cell losses in the MS, as indicated by decreased choline acetyltransferase-immunoreactivity in the medial septum. Daily administrations of YMjd (100 mg/kg, i.p.) for 21 consecutive days produced significant reversals of ibotenic acid-induced deficit in learning and memory. These treatments also reduced the loss of choline acetyltransferase (ChAT) immunoreactivity in the MS induced by ibotenic acid. These results suggest that impairments of spatial learning and memory might be attributable to the degeneration of septohippocampal cholinergic (SHC) neurons and that YMjd treatment ameliorated learning and memory deficits partly due through neuroprotective effects on the central acetylcholine system. Our studies suggest that YMjd might be useful in the treatment of Alzheimer’s disease.

Key words Yukmijihwang-tang derivative; Morris water maze test; Alzheimer’s disease

The consistent findings in Alzheimer’s disease (AD) patients are impairment in cognitive performances such as attention, learning and memory, and change of cholinergic markers, including levels of acetylcholine (ACh) and choline acetyltransferase (ChAT). Since cholinergic deficits are found to be associated with cognitive decline or dementia, increase of the central cholinergic system by selective inhibition of cholinesterase is one of the more promising current therapeutic tools for treating AD. Cholinergic neurons originating in the nucleus basalis of Meynert (NBM) and the medial septum (MS) project to areas such as the cortex and hippocampus, where play the role of ACh in cognition. Lesioning these pathways in rodents decreases ACh release and produces an impairment of memory-related task performance. The observations that impairment can be reversed by cholinergic agonists suggest that the cholinergic system is damaged in AD and that drugs which induce cholinergic activity may treat AD. For the testing of putative, cognition-enhancing agents, the establishment and standardization of animal cognitive deficit models are required. Lesion of the MS in animals has been widely approved as an animal model of memory loss. Cognitive dysfunctions after lesioning the MS are mainly considered to be due to deafferentation of the hippocampus, which plays a major role in learning and memory. Coordinated action of acetylcholine and glutamate is important for the production of memory. Acetylcholine (ACh) facilitates glutamate activity by coordinating the states of acquisition and recall in the cortex and hippocampus and the activation of N-methyl-D-aspartate (NMDA) receptors is a prerequisite for the stimulation of long-term potentiation (LTP) in the septohippocampal cholinergic system, which is involved in the memory performance of rats in some learning tasks that depend on hippocampal functions. Ibotenic acid stimulates neuronal necrosis by a hyperstimulation of the NMDA glutamate receptors leading to calcium overload. Its excitotoxic properties are confined to somata of neurons and therefore axons and blood vessels that course through the target areas remain intact. Injections of ibotenic acid into the MS in rats result in a significant deficit in the Morris water maze, which has been designed to measure spatial learning and memory, and provide a more advanced stage of the neurodegeneration as an animal model of AD. Herbal medicines, such as Ginkgo biloba, Ginseng, or Melisa officinalis, have been commonly used as memory or cognition enhancers. The effects of these enhancers have been demonstrated scientifically. Yukmijihwang-tang (YMJ or Luweihuang-wang) is another memory or cognition enhancer. YMJ is composed of 6 herbal medicines, including steamed Rehmannia radix, Discoraea radix, Corni fructus, Hoelen, Mountain cortex radicis, and Alismatis radix. YMJ has long been applied in the treatment of diabetes mellitus and neurosis. Ancient Chinese herbal textbooks also refer to YMJ as an anti-aging prescription. A series of modern medical reports show that YMJ has anti-aging effects in vivo and in vitro. In addition, antioxidant and free radical scavenging activities of YMJ have been clinically demonstrated. Besides its anti-aging effects, Wei have reported that YMJ prevents the deterioration of learning and memory ability in senescent accelerated mice and hydrocortisone-treated mice. It is also reported that YMJ reverses scopolamine-induced and p-chloroamphetamine-induced amnesia in rats. In addition, our unpublished data shows that YMJ derivatives (YMjd), including Lycii fructus, enhance memory retention by protecting neuronal cells from attack by reactive oxygen species (data not shown). Our previous results have revealed that YMjd has a significant effect on memory enhancement and the expression of genes associated not only with the prevention of neuronal degeneration but also with neuronal...
growth events. Recent double-blind placebo-controlled trials have also demonstrated that YMJD significantly enhances cognitive abilities in normal human subjects. However, there have been few reported studies on accessing learning and memory enhancement triggered by treatment with YMJD in dementia animal models. Thus, we examined the effect of YMJD on learning and memory ability in ibotenic acid-induced amnesia rats using the Morris water maze, and the relationship between the cholinergic marker in the MS and the neural mechanism underlying its improving effect on memory is discussed.

MATERIALS AND METHODS

Animals Ten-week old male Sprague-Dawley rats weighing 270 to 290 g were obtained from Samtaco Corp. (Kyangki-do, Korea). The experimental procedures were carried out according to the animal care guidelines of the NIH and the Kyung Hee University Institutional Animal Care. The rats were housed, and allowed free access to their diets and tap water, under controlled and restricted pathogen-free conditions (room temperature: 23±3 °C, relative humidity: 50±10%, light cycle: 07:00—19:00). The experiments began at least 7 d after the rats’ arrival in individual home cages.

Preparation of YMJD All dried water extracts of the YMJD components used in the present study were purchased from the Sun-Ten Pharmaceutical Company (Taiwan). The prescription and the ratio of each component in YMJD are shown in Table 1.

Lesioning Procedure and Administration of YMJD The general procedures for surgery were the same for each group, except that artificial cerebrospinal fluid (CSF) was microinjected into the MS in the sham group (Control), whereas ibotenic acid (Sigma, St. Louis, MO, U.S.A.) was microinjected into the rats in the lesion group at a concentration of 4 μg/μl of CSF. The anesthetized rat under pentobarbital (50 mg/kg, i.p.) was placed in a stereotaxic apparatus. The skin over the rat’s skull was shaved and cleaned with betadine, an incision was made through the skin and muscle to expose the skull, and the skin was retracted. Two holes were drilled using stereotaxic coordinates based on the Paxinos and Watson brain atlas in the MS (AP: −0.2, L: ±0.3, DV: −6.2 referenced to the bregma). A 22-gauge Hamilton syringe (Reno, NV, U.S.A.) filled either with artificial CSF or ibotenic acid was slowly infused at 0.02 μl/min using a microinjection pump (Pump 22; Harvard Apparatus, South Natick, MA, U.S.A.) for 5 min and the syringe was left for a further 5 min. YMJD was dissolved in saline (100 mg/ml). The day after surgery, a suspension of YMJD or saline was administered intraperitoneally for 3 weeks. In the experiment, the sham (Control) and ibotenic acid lesioned (IA) received saline (1 ml/kg per day), and the ibotenic acid lesioned + YMJD group (IA-Y) received YMJD (100 mg/kg per day).

Morris Water Maze Test The water maze was a circular pool (painted white, 180 cm in diameter, 50 cm high) constructed from fiberglass. The pool was filled up to 30 cm high with water that was maintained at a temperature of 22±2 °C. The pool was divided into four quadrants, with four starting locations, north (N), east (E), south (S), and west (W), at equal distances from the rim. During testing in the water maze, a transparent platform, 12 cm in diameter, was located 1.5 cm below the water at the center of the northeast quadrant in the pool, approximately 45 cm from the sidewalls. The pool was surrounded by many cues external to the maze; these were visible from the pool and could be used by the rats for spatial orientation. The position of the cues remained unchanged throughout training.

A videocamera was mounted to the ceiling above the pool and was connected to a videorecorder and tracking device (SMART; Pan-Lab, Barcelona, Spain), which permitted online 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 s and the latency to find the submerged platform was recorded. The animals were tested in this way for 6 d, and then received a probe trial on the 7th day. For the probe trial, the platform was removed from the pool and the animal was then released from the quadrant opposite to where the platform would have been located. The length of time of the trial was 60 s, after which the rat was removed from the pool. The proportion of time that the rat spent searching for the platform in the training quadrant; i.e., the previous location of the platform was recorded and used as a measure of retention: the swim time and distance spent in each pool quadrant was video-recorded for later calculation of the percentage of time spent in each quadrant.

ChAT Immunohistochemistry At the end of the behavioral observation, the rats were deeply anesthetized with a sodium pentobarbital (100 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-buffered saline (PBS). The brains were removed, post-fixed overnight, and cryoprotected in 20% sucrose with PBS. The brains were cut by a cryostat as 30-μm coronal sections, which were processed immunohistochemically as free-floating sections. The sections were washed in PBS containing

<table>
<thead>
<tr>
<th>Herbal medicines</th>
<th>Ratio (%)</th>
<th>Standard materials (SM)</th>
<th>SM contents (mg/YMJD g ext)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rehmanniae radix Preparat</td>
<td>4 (26.5%)</td>
<td>5-HMF</td>
<td>0.20±0.02 (0.02%)</td>
</tr>
<tr>
<td>Corni fructus</td>
<td>2 (13%)</td>
<td>Logamin</td>
<td>1.29±0.05 (0.13%)</td>
</tr>
<tr>
<td>Lycii fructus</td>
<td>4 (26.5%)</td>
<td>Betain</td>
<td>0.32±0.02 (0.03%)</td>
</tr>
<tr>
<td>Discordiae radix</td>
<td>2 (13%)</td>
<td>Allantoin</td>
<td>1.31±0.21 (0.13%)</td>
</tr>
<tr>
<td>Hoelen</td>
<td>1 (%)</td>
<td>Paeonol</td>
<td>0.93±0.033 (0.09%)</td>
</tr>
<tr>
<td>Mountain cortex radix</td>
<td>1 (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alismatis radix</td>
<td>1 (%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
with the platform among groups differed ($p<0.05$) and Control and IA-Y groups spent more distance in the pool quadrant with the platform than the IA group ($p<0.01$ for the Control group, $p<0.05$ for the IA-Y group, $F_{(2,14)}=9.186$ see Fig. 3). These results suggest that ibotenic acid lesion severely impaired spatial cognition in the water maze task, and the YMJd treatment group attenuated ibotenic acid-induced learning and memory damage in the water maze.

### ChAT Immunohistochemistry

The results of the ChAT immunoreactive analysis in the MS are shown in Figs. 4, 5. The number of neurons of ChAT was 13.0±0.9 (mean±S.E.M.) in the control group and 8.5±0.9 in the IA group. This reduction was approximately 35% and the IA-Y group had significant recovering effect on the number of neurons of ChAT compared to the IA group ($p<0.001$, $F_{(2,28)}=56.11$). The Tukey post-hoc test revealed that the IA-Y significantly reversed the reduced number of ChAT neurons induced by ibotenic acid, shown in the IA group ($p<0.001$ in the MS area, $F_{(2,18)}=56.11$). Ibotenic acid lesion severely impaired the neurons of ChAT, an acetylcholine producing enzyme and the YMJd treatment group attenuated ibotenic acid-induced ChAT neuron damage in the MS. The YMJd treatment group may have attenuated ibotenic acid-induced learning and memory damage in the water maze through the recovery of the ibotenic acid-induced ChAT neuron damage.

### RESULTS

#### Morris Water Maze Test

The results of acquisition of the Morris water maze task are depicted in Fig. 1 and Table 2. The escape latency differed among the groups when averaged over all sessions ($p<0.05$). Post hoc comparisons revealed that the ibotenic acid lesion group (IA) needed more time to locate the platform than the IA-Y group did. During the experiment, the latency to reach the platform diminished ($p<0.01$) over the time, but there was no interaction between the group and the day ($p>0.05$). Tukey post-hoc test revealed that the IA-Y ($p<0.01$ $F_{(2,17)}=14.19$ on day 6) significantly reduced the latency of swimming time to reach the platform, compared with those of the IA. In analysis of the performance on the probe trial, the percentage of time and distance spent swimming in the pool quadrant with platform is illustrated in Figs. 2, 3. The time spent in the pool quadrant with the platform among groups differed ($p<0.05$) and Control and IA-Y groups spent more time in the pool quadrant with the platform than the IA group ($p<0.01$ for the Control group, $p<0.05$ for the IA-Y group, $F_{(2,17)}=9.314$, see Fig. 2). On the other hand, the distance spent in the pool quadrant among groups followed by the Tukey test.}

### Table 2. Comparison of Acquisition Performance on the Morris Water Maze Task among the Three Groups of Rats

<table>
<thead>
<tr>
<th>Day</th>
<th>Control Mean</th>
<th>Control S.E.M.</th>
<th>IA Mean</th>
<th>IA S.E.M.</th>
<th>IA-Y Mean</th>
<th>IA-Y S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>113.3</td>
<td>29.7</td>
<td>145.4</td>
<td>10.6</td>
<td>127.1</td>
<td>33.8</td>
</tr>
<tr>
<td>2</td>
<td>99.2</td>
<td>35.6</td>
<td>108</td>
<td>14.7</td>
<td>107.2</td>
<td>42.1</td>
</tr>
<tr>
<td>3</td>
<td>77.2</td>
<td>35.7</td>
<td>101.3</td>
<td>17.6</td>
<td>97.2</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>38.3</td>
<td>21.7</td>
<td>106.5</td>
<td>12.4</td>
<td>92.5</td>
<td>23.3</td>
</tr>
<tr>
<td>5</td>
<td>24.8</td>
<td>7.2</td>
<td>77.7</td>
<td>14.9</td>
<td>60.8</td>
<td>39.8</td>
</tr>
<tr>
<td>6</td>
<td>47.2</td>
<td>18.5</td>
<td>67.7</td>
<td>16.1**</td>
<td>34.6</td>
<td>12.3**</td>
</tr>
</tbody>
</table>

Mean swimming time (s) traveled per trial. Mean values of the four trials per day for 6 d for each of the three groups are shown. Repeated measures of ANOVA of swimming time among the groups followed by the Tukey test. $\ast p<0.01$, as compared with the corresponding data of control group; $\ast\ast p<0.01$, as compared with the corresponding data of the IA group.
DISCUSSION

The relevant animal model of AD is an important research strategy in ongoing trials to understand the pathology and therapeutics of AD. Although no current model develops the full pathologic spectrum of this disease, injection of excitotoxin into the MS has been shown to damage memory and induce a degree of Alzheimer-type neurodegeneration.9) Ibotenic acid, a rigid structural analogue of glutamate, is a neuroexcitatory compound and is also a pharmacologic method used for studies of rat models involving lesion of cholinergic neurons by stereotaxic injections into the brain.24,25) After ibotenic acid-induced lesions to the MS, at the source of the hippocampal branches of the forebrain cholinergic projection system, rats showed long-lasting stable impairment in reference and working memory in both spatial (place) and in associative (cue) radial maze tasks.9,10) Injections of ibotenic acid into the MS or the nucleus basalis of Meynert (NBM) in rats produced decreased activities of ChAT in the hippocampus and frontal cortex, respectively, followed by impairment in memory acquisition.26) Consistent with the previous reports, the present study demonstrated that the injection of ibotenic acid produced a loss of spatial working memory and cholinergic markers as indicated by the reduction of ChAT reactive neurons in MS. Cognitive dysfunctions after lesioning the MS are mainly considered to be due to deafferentiation of the hippocampus, which plays a major role in learning and memory.11,12) The Morris water maze task used to test relatively pure spatial learning capability and reference memory can determine whether cholinergic depletion is sufficient to produce memory impairment.27) In
the current study, injections of ibotenic acid into the MS in rats affected the performance of rats in the water maze. The IA group showed poorer performance of acquisition and retention test than did the control group. The latencies to find the platform on acquisition trials by the YMJd-pretreated group were significantly decreased compared to those of the IA group. The YMJd treatment group also spent a greater proportion of the probe trial searching in the training quadrant, demonstrating that treatment with YMJd for 3 weeks attenuated ibotenic acid-induced learning and memory deficits in the Morris water maze. Treatment of Bai Wei Dihung Wan, a traditional chinese medicine that consists of YMJ and two other medicinal herbs give pharmacological evidence of an anti-dementia effect. The clinical efficacy of Bai Wei Dihung Wan in patients with dementia has been described by a double blind and placebo controlled study.28) **Mountain cortex radicis**, a component of YMJd decreased ROS generation and cytotoxicity in hydrogen peroxide stimulated neuronal cells through gene expressions of heme oxygenase (HO) and COMT, which play a major role in regulating ROS production.29) It was shown that **Rehmannia radix**, a component of YMJd improved the function of learning and memory of monosodium glutamate (MSG) treated rats through anti-oxidation and the increase of the expression of hippocampal c-fos and NGF and intelligence in human.30,31) Also, it was reported that **Lycii Fructus** and **Corni fructus**, components of YMJd presented a strong anti-oxidative effects.2,3) In addition to such anti-oxidant effects from some components of YMJd, it was found that **Lycii Fructus**, **Rehmannia radix**, **Discorea radix**, **Corni fructus**, and **Mountain cortex radicis**, components of YMJd showed anti-inflammatory effects. Thus, such anti-oxidative and anti-inflammatory effects from those components in YMJd may have been responsible for the protection against ibotenic acid-induced cell damage and cognitive deficits shown in the present study. Future studies are needed to understand the neuroprotective and behavioral effects of these components in the ibotenic acid lesion model. In the present study, we found that the IA group had a reduction in ChAT activity in the MS. It may suggest that the reduction in MS cell loss and recovery of reduced ChAT activity in the MS after treatment with YMJd might be associated with the improvement of learning and memory since the IA-Y group had a greater cholinergic

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REFERENCES