The Effects of Acupuncture at Sobu (HT8) and Haenggan (LR2) on Scopolamine-induced Cognitive Impairment in Rat Model

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ABSTRACT

Background: This study investigated the effects of acupuncture at Sobu (HT8) and Haenggan (LR2) on scopolamine-induced, cognitively impaired rats.

Methods: Scopolamine-treated Sprague-Dawley rats were divided into 6 groups; normal, control, HT8, LR2, HT8 + LR2 and sham group. Cognitive impairment was induced by scopolamine, in control, and then in HT8, LR2, HT8 + LR2 and sham groups. Acupuncture treatment was performed at HT8, LR2, HT8 + LR2, and a random acupoint, respectively, every other day for 2 weeks. After each treatment, behavior change was observed and the rats were sacrificed. The change in brain-derived neurotrophic factor, glutathione peroxidase, and superoxide dismutase activity was evaluated by polymerase chain reaction.

Results: Latency time to target in Morris Water-Maze test for the HT8 + LR2 group showed a significant decrease compared with control (p<0.05). Target crossing times and time zone ratios in Morris Water-Maze test for HT8 + LR2 group showed a significant increase compared with control (p<0.01). In the Y-Maze test the HT8 + LR2 group showed a significant increase compared with control (p<0.05). Brain-derived neurotrophic factor, glutathione peroxidase, and superoxide dismutase, in the HT8 + LR2 group, showed a significantly increased level compared with control (p<0.05). Neural activity of acetylcholine esterase in HT8 + LR2 group showed a significant decrease compared with the control group (p<0.01), choline acetyltransferase activity in the HT8 + LR2 group showed a significant increase compared with control (p<0.05).

Conclusion: Acupuncture at HT8 + LR2 restored scopolamine-induced cognitive impairment, suggesting acupuncture could be an alternative to improve cognitive function.

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Introduction

Due to recent industrial and scientific advances in society, the quality of human life has improved, and the average life expectancy has increased. Consequently, the population of elderly has also increased. Dementia has received much attention as an age-related disease [1]. However, dementia does not occur in all elderly people, and since mild cognitive impairment (MCI) with deterioration in cognitive function and memory are known to precede dementia [2], the prevention and treatment of MCI are considered important.

MCI refers to deteriorated memory, amongst various other cognitive functions, compared to others in a similar age group, cognitive changes between unaffected individuals and those with dementia appear as a gradual deterioration in cognitive function [1]. MCI is known to be more common among males [3], and as of 2008, the prevalence of MCI in Korea was reported to be 24.1%, accounting for approximately 25% of the elderly population who are 65 years and older [4].

In Korean medicine (KM), MCI has been studied to date from the amnestic perspective in relation to cognitive impairment. This includes the study by Kim et al [5] on non-drug therapy for MCI. Another study by Kim et al [6] and one by Choi et al [7], targeted the Sobu (HT8) and Haenggan (LR2) during treatment. However, the aforementioned studies were related to heart rate variability and obesity, there are currently no studies related to cognitive impairment. Moreover, although there are studies on improvement in scopolamine-induced deterioration in memory and cognitive function, studies related to the Sobu (HT8) and Haenggan (LR2)
were not found. In the present study, Sobu (HT8) and Haenggan (LR2) were used to investigate whether acupuncture at these points could improve scopolamine-induced cognitive impairment in white rats. Moreover, since KM emphasizes the importance of the ideology behind preventive medicine, “handle it before it becomes a disease,” [8] it was determined that treating MCI, which represents a pre-stage of dementia, would be important for studying dementia. Consequently, in the present study, rats were injected with scopolamine to induce cognitive impairment during the performance of the Morris Water-Maze test and the Y-Maze test. The study also investigated acetylcholine esterase (AChE) and choline acetyltransferase (ChAT) neural activity in the hippocampus and measured changes in brain-derived neurotrophic factor (BDNF), glutathione peroxidase (GPx), and superoxide dismutase (SOD) using polymerase chain reaction (PCR).

Materials and Methods

Animals

In this study, 8-week-old male Sprague Dawley rats, weighing approximately 230~250 g were used (Samtako, Korea). The rats were kept in a breeding room with constant temperature and humidity (indoor temperature of 24 ± 1°C and humidity of 60 ± 5%), and were given ad libitum access to solid food (Pellet, Samtako, Korea) and water. Experiments on rats began after 1 week of acclimatization (2 Jun 2017, IACUC, Dongshin Univ.).

Acupuncture

The disposable needles used for acupuncture were 0.25 mm in diameter and 15 mm in length (Dongbang Medical, Korea).

Acupoint

Acupoints between the 4th and 5th metacarpal bone of both the upper limbs bottom side corresponding to Sobu (HT8) and between the first and second metatarsal bone of both the upper sides of lower limbs corresponding to Haenggan (LR2) were used [9,10].

Scopolamine-induced cognitive impairment

Scopolamine (SIGMA, USA) was dissolved and diluted in saline to a final concentration of 5 mg/kg, and was intraperitoneally injected around umbilicus in each rat a total of 6 times, once every other day, without the use of anesthesia.

Classification of experimental groups

Experimental groups comprised the normal group (Normal, n = 5) which did not receive any treatment, control group (Control, n = 5), Sobu acupuncture treatment group (HT8, n = 5), Haenggan acupuncture treatment group (LR2, n = 5), simultaneous Sobu and Haenggan treatment group (HT8 + LR2, n = 5), and sham group (Sham, n = 5) which received acupuncture in both gluteal areas.

The treatment was administered after the rats underwent inhalation anesthesia using 5% isoflurane (Hana Pharm, Korea) in 80% O2 gas, for a total of 6 times, once every other day, starting from the day of induction with scopolamine (Table 1).

Table 1. Classification of Experimental Groups.

<table>
<thead>
<tr>
<th>Group (n = 5)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>not SCI induction, treated</td>
</tr>
<tr>
<td>Control</td>
<td>SCI induction, not treated</td>
</tr>
<tr>
<td>HT8</td>
<td>SCI induction, treated at HT8</td>
</tr>
<tr>
<td>LR2</td>
<td>SCI induction, treated at LR2</td>
</tr>
<tr>
<td>HT8 + LR2</td>
<td>SCI induction, treated at HT8 with LR2</td>
</tr>
<tr>
<td>Sham</td>
<td>SCI induction, treated at random acupoint</td>
</tr>
</tbody>
</table>

Morris Water-Maze test

The Morris Water-Maze test (MWM) was used to assess spatial learning and memory ability. A circular water tank was filled with water to a height of 22 cm. The water temperature was maintained at 24 ± 1°C. The entire experimental space was divided into 4 quadrants and illuminated indirectly. An escape platform was placed inside the water tank and the behavior of rats was analyzed using a tracking program (SMART, PanLab, Spain).

The MWM was performed once a day for 3 days, starting 12 days after scopolamine injection. Training was conducted 3 times per session. The water tank was divided into 4 quadrants and the escape platform was placed in the same quadrant from the first to third trial. The brim of the water tank was marked with safeguards. A 30-second inter-training interval was given between training sessions. With each session, the rat was placed at a different starting point. On the first day of the training sessions, if the rat was unable to find the escape route after 1 minute, it was placed on the escape platform. This was done to allow the rat to observe the surroundings for 15 seconds before it learned the location of the platform. The training recommenced after 30 seconds of rest. In cases where the rat was able to find and climb the escape platform within 1 minute, it was allowed to observe the surroundings from the escape platform for 15 seconds, and training recommenced after 30 seconds of rest.

The target crossing test was performed the day after the third session was completed (the 12th day after treatment). After removing the escape platform, the rat was placed inside the circular water tank and its movement was monitored for 2 minutes. In this test, we noted the number of times the animal passed through the location where the escape platform used to be, the length of time spent in the quadrant where the escape platform used to be, and the amount of time spent to locate the quadrant where the escape platform used to be.

Y-Maze test

In the Y-Maze test, the maze was comprised of 3 black acrylic arms that formed a Y-shape. The 3 arms were labeled A, B, and C. The rat was placed in the center of the maze and allowed to move freely for 6 minutes, after which the path the rat used to enter an arm was examined. Measurements were limited to cases where the tail of the rat completely entered the arm. Cases where the same arm was re-entered were also recorded.

In the Y-Maze test, measurements were made on the 11th day after scopolamine was induced, and a score of one point was assigned when the 3 different arms were entered in order (ABC, BCA, and CAB; actual alternation), while alternation behavior was
measured using the total arm entry score.

\[
\text{Maximum alternation} = \frac{\text{Total number of arm entry}}{2} - \text{Actual alternation} \times 100
\]

**Total RNA isolation and RT-PCR analysis**

Total RNA isolation

The hippocampus was isolated from the retrieved brain for rapid freezing using liquid nitrogen and stored at −70°C until needed for analysis.

For total RNA isolation, 800 µl of Trizol Reagent (Life technologies, USA) was added to the retrieved hippocampus and homogenized in Precellys 24 (Bertin technologies, France). Afterwards, 200 µl of chloroform (Sigma, USA) was added to the homogenized solution. The mixture was shaken and mixed for 15 seconds, after which it was left for 5 minutes at room temperature and later centrifuged (Centrifuge 5415 R; Eppendorf, Germany) for 5 minutes (4°C and 14,000 rpm) to remove any cellular residues. After adding 500 µl of isoamyl alcohol (Sigma, USA) to the supernatant obtained from centrifugation, the mixture was left for 5 minutes at room temperature, and then centrifuged for 8 minutes (4°C and 14,000 rpm) to obtain the RNA pellets. The pellets obtained from centrifugation were placed in DEPC (diethylpyrocarbonate), together with 70% ethanol that was cold-stored, and centrifuged for 5 minutes (4°C and 7,500 rpm). Subsequently, all components other than the pellets were removed, and the residual ethanol was left to dry at room temperature for 5 minutes; the pellet was then dissolved in DEPC-treated water and OD_{260} value was measured using a spectrophotometer (Biophoto meter, Eppendorf, Germany) to quantify the concentration and purity of RNA.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

5 µg of isolated total RNA were added to the RT premix (Bioneer, Korea), together with 2.5 µl of oligo (dT), DEPC-treated water. The Mastercycler gradient (Eppendorf, Germany) was used to synthesize 50 µl of cDNA for use as the template for PCR amplification. Here, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense primer: 5'-ACTCCATCACCATCTTCCAG-3', antisense primer: 5'-CCTGCTTTACCACCTCCTTG-3') was used as the internal control. The reverse transcription temperature cycle included 1 hour of cDNA synthesis at 42°C, 5 minutes of denaturing, and 5 minutes of cooling at 4°C. The polymerase chain reaction involved placing cDNA, 10 ng sense primer, 10 ng antisense primer, and DEPC-treated water in the PCR premix (Bioneer, Korea) and using the Mastercycler gradient (Eppendorf, Germany) for amplification. The PCR temperature cycle included 300 seconds of pre-denaturation at 95°C for amplification of cDNA, 40 seconds of melting at 94°C, 40 seconds of annealing at 55°C, and 90 seconds of extension at 72°C. This process was repeated for 34 cycles. In the final cycle, extension was performed for 600 seconds at 72°C.

Gene amplification of Cu-Zn SOD, GPx, and BDNF were performed using primer (sense primer: 5'-GCAGAAGGGCAGGGTTG AAC-3', antisense primer: 5'-TAGGACACACAGATGAGT-3'), primer (sense primer: 5'-CTCTCCGGCGGTTGACAGT-3', antisense primer: 5'-CCACCCCGGGTTCGAGCT-3'), and primer (sense primer: 5'-CAGGGGCATAGACAAAAG-3', antisense primer: 5'-CTCTCCGCGGTGGCACAGT-3'), respectively. The amplified BDNF, GPx, and Cu-Zn SOD DNA were electrophoresed at 100 V using 0.5x TBE buffer (80mM Tris-HCl, 80mM boric acid, 2mM EDTA, pH 8.3) on 1.5% agarose gel that contained Greenview nucleic acid gel stain (IO Rodeo, 1:10,000). Afterwards, Image Station (Samsung, Korea) was used for imaging and the Alphalase FC Stand Alone Software (Alpha Innotech, USA) was used for measurement.

**Immunohistochemistry**

Upon completion of all experiments, the animals were anesthetized by inhalation anesthesia using 80% O2 gas and 5% isoflurane (Hana Pharm, Korea), after which 200 ml of 0.9% saline was perfused through the heart, followed by 200 ml of 5% formalin prepared with phosphate buffer. After fixation, the brain was retrieved from the rat and fixed with 10% formalin solution (fixative), after which it was placed in phosphate buffered saline (PBS) containing 30% sucrose and stored for a day at 4°C. The following day, the brain was rapid-frozen and 30 µm thick sections of the brain tissue containing the hippocampus were cut using a Cryotome (Shandon, Japan).

Tissues that had been stained were observed using the 40x objective of an optical microscope (Nikon, Japan), while the density of neurons was measured using Scion image program (Scion Corp. MD, USA).

**Acetylcholine Esterase Neural Activity**

The brain tissue was initially washed 3 times with 0.1M PBS, and after 40 minutes of blocking with 2% rabbit serum, AChE antibody 1:200 (Santa Cruz, USA), was added to each sample. The primary antibody was prepared by a 300-fold dilution with 0.1% sodium azide (Sigma, USA) buffer in 0.1M PBS. The brain tissue was incubated in primary anti-serum for 72 hours at 4°C. Subsequently, the brain tissues were washed with 0.1M PBS, at least 3 times, and then incubated with biotinylated universal secondary antibody (Quick Kit, Vector Laboratories, USA) for 40 minutes at 37°C. After washing again with 0.1M PBS, at least 3 times, the brain tissue was soaked in streptavidin peroxidase preformed complex (Quick Kit, Vector Laboratories, Burlingame, CA, USA) for 40 minutes at 37°C. After washing again with 0.1M PBS, the brain tissue was incubated in primary anti-serum for 72 hours at 4°C. Subsequently, the brain tissues were washed with 0.1M PBS, at least 3 times, and stained with diaminobenzidine (DAB), (Vector Laboratories, USA), a coloring agent. The DAB color development was stopped using 0.1M PBS.

**Choline acetyltransferase neural activity**

The brain tissue was initially washed 3 times with 0.1M PBS, and after 40 minutes of blocking with 2% rabbit serum, ChAT antibody 1:250 (Abcam, UK), was added to the brain tissue. The primary antibody was prepared by a 400-fold dilution with 0.1% sodium azide (Sigma, USA) buffer in 0.1M PBS. The tissues were then incubated in primary anti-serum for 72 hours at 4°C. Subsequently, the brain tissue was washed with 0.1M PBS, at least 3 times, and incubated with biotinylated universal secondary antibody (Quick Kit, Vector Laboratories, Burlingame, CA, USA) for 30 minutes at 37°C. After washing again with 0.1M PBS, at least 3 times, the brain tissue was soaked in streptavidin peroxidase preformed complex (Quick Kit, Vector Laboratories, Burlingame, CA, USA) for 30 minutes at 37°C. The brain tissues were washed again with 0.1M PBS, at least 3 times, and stained with diaminobenzidine (DAB), (Vector Laboratories, USA), a coloring agent. The DAB color development was stopped using 0.1M PBS.

**Statistical analyses**

All measured values were expressed as means ± SD with Excel.
Statistical analysis was performed using the SPSS for Windows (SPSS, ver. 21, USA). The Mann-Whitney U test, a non-parametric method, was used for statistical analysis between experimental groups. Statistical significance was tested for each experimental group with $\alpha = 0.05$ ($p < 0.05$) and $\alpha = 0.01$ ($p < 0.01$) relative to the control group.

Results

Effects of Morris Water-Maze test

Latency time to target

Latency time was significantly higher in the control group ($p < 0.01$) 64.9 ± 14.9 seconds than in the normal group 7.1 ± 1.8 seconds. The HT8, LR2, HT8 + LR2, and sham groups had latency times of 22.2 ± 8.1, 29.1 ± 13.3, 21.1 ± 5.3, and 47.8 ± 21.7 seconds, respectively. The latency for the HT8 + LR2 group was significantly lower than that for the control group (Fig. 2).

Target crossing times

The number of target crossings was significantly lower in the control group (3.4 ± 0.60 times, $p < 0.05$) than in the normal group (7.0 ± 1.26 times). The number of target crossings for the HT8, LR2, HT8 + LR2, and Sham groups were 6.0 ± 1.47, 5.5 ± 0.87, 7.5 ± 0.65, and 4.3 ± 0.85 times, respectively. The number of target crossing for the HT8 + LR2 group was significantly higher than that for the control group (Fig. 3).

Time zone ratio

The time zone percentage was significantly lower in the control group (15.3 ± 1.9%, $p < 0.05$) than in the normal group (27.7 ± 4.7%). The HT8, LR2, HT8 + LR2, and Sham groups had time zone percentages of 20.4 ± 0.5%, 25.8 ± 2.7%, 27.8 ± 2.8%, and 20.3 ± 3.5%, respectively. The time zone percentage of the LR2 and HT8 + LR2 groups were significantly higher than that for the control group (Fig. 4).
Effects of Y-Maze test

Alternation behavior was significantly lower in the control group (34.1 ± 6.3%, p < 0.05) than in the normal group (52.8 ± 2.6%). The alternation behavior of rats in the HT8, LR2, HT8 + LR2, and Sham groups were 49.2 ± 2.6%, 50.1 ± 6.1%, 55.7 ± 2.5%, and 44.1 ± 4.4%, respectively, and the alternation behavior in the HT8 + LR2 group was significantly higher than that in the control group (Fig. 5).

Effects of brain-derived neurotrophic factor, glutathione peroxidase, and superoxide dismutase expression by PCR

Brain-derived neurotrophic factor

The OD value for BDNF was significantly lower in the control group (91.5 ± 2.2, p < 0.05) than in the normal group (113.8 ± 2.5). The OD values for the HT8, LR2, HT8 + LR2, and Sham groups were 105.3 ± 6.8, 110.5 ± 12.5, 111.1 ± 2.2, and 100.4 ± 8.6, respectively, while that for the HT8 + LR2 group was significantly higher than that for the control group (Fig. 6).

Glutathione peroxidase

The OD value for GPx was significantly lower in the control group (133.8 ± 4.6, p < 0.01) than in the normal group (156.1 ± 4.2). The HT8, LR2, HT8 + LR2, and non-AP groups had OD values for GPx of 148.6 ± 7.6, 146.8 ± 7.9, 159.0 ± 7.4, and 145.6 ± 8.1, respectively. The OD value of GPx for the HT8 + LR2 group was significantly higher than that for the control group (Fig. 7).

Superoxide dismutase

The OD value for SOD was significantly lower in the control group (133.4 ± 2.6, p < 0.05) than in the normal group (145.2 ± 3.2). The HT8, LR2, HT8 + LR2, and non-AP groups had OD values for SOD of 143.5 ± 5.3, 146.9 ± 5.5, 148.1 ± 5.7, and 141.8 ± 8.7, respectively. The HT8 + LR2 group had a significantly higher OD value of SOD than the control group (Fig. 8).

**Fig. 5.** HT8 + LR2 group showed a significant increase compared with control in the Y-Maze test.

Values are presented as mean ± SE.

* *p < 0.05 compared with normal; † *p < 0.05 compared with control.

**Fig. 6.** BDNF in the HT8 + LR2 group, showed a significantly increased level compared with control.

Values are presented as mean ± SE.

* *p < 0.05 compared with normal; † *p < 0.05 compared with control.

**Fig. 7.** GPx in the HT8 + LR2 group, showed a significantly increased level compared with control.

Values are presented as mean ± SE.

* *p < 0.05 compared with normal; † *p < 0.05 compared with control.

BDNF, brain-derived neurotrophic factor; HT8, Sobu; LR2, Haenggan; SCI, scopolamine-induced
Acetylcholine esterase neural activity

The activity index of AChE neural activity was significantly higher in the control group (6.4 ± 0.97, p<0.01) than in the normal group (2.5 ± 0.09). The activity index of AChE in the HT8, LR2, HT8 + LR2, and non-AP groups were 4.5 ± 0.59, 3.4 ± 0.88, 2.5 ± 0.38, and 5.7 ± 0.72, respectively, while that for the HT8 + LR2 group was significantly lower than that for the control group (Fig. 9).

Choline acetyltransferase neural activity

Observations on the changes in ChAT neural activities after Sobu and Haenggan acupuncture showed that the activity index was significantly lower in the control group (2.4 ± 0.49, p<0.01) than in the normal group (5.2 ± 0.46). The activity indices of the HT8, LR2, HT8 + LR2, and non-AP groups were 3.0 ± 0.36, 3.8 ± 0.12, 4.1 ± 0.24, and 2.8 ± 0.09, respectively. The HT8 + LR2 group had an activity index which was significantly higher than that for the control group (Fig. 10).

Discussion

MCI is considered a precursor to dementia. Amongst all patients who are diagnosed with MCI for the first time, approximately 50% of those patients will see their condition progress into Alzheimer's disease within 4 years [11], whilst also being at risk for advancing to dementia with the chief complaint being memory impairment [12]. Petersen et al [13] suggested that MCI alone cannot be classified as a typical dementia syndrome or a specific disease but suggested that it is a characteristic of a clearly objective cognitive impairment. Hwang et al [14] reported that MCI patients display a higher frequency of aberrant behaviors, such as hypersensitivity, irritability, anxiety, and depression compared with unaffected people. In the majority of cases, symptoms worsen each year and MCI, in 12% of the elderly, progresses to Alzheimer's disease or other forms of dementia [15], which represents a greater
progression than observed amongst an unaffected population. Since MCI increases the morbidity of dementia, measures for its prevention are needed [12].

From an amnestic perspective, KM suggests MCI as a condition with symptoms such as being oblivious to one’s own words or actions, starting a task but not finishing it, and not fully recognizing the beginning and the end of a conversation. A study by Kim et al [16] showed KM-based treatments for MCI and dementia, that used Sinmun (HT7), Baekhoe (GV20), Simsu (BL15), Sosang (LU11), Gansa (PC5), Naegwan (PC6), Sobu (HT8), and Haenggan (LR2) for treating forgetfulness and dementia, whilst a study by Shin et al [17] reported that acupuncture on Sobyu (HT8) and Haenggan (LR2) had an effect on regulation of regional cerebral blood flow. Acupuncture on HT8 and LR2 appears to have an effect not only on dementia, but also on vascular dementia as a result of effects on cerebrovascular lesions. It was also determined that dementia-related diseases that accompany cognitive impairment are deeply associated with depression, and thus, HT8 and LR2 were designated as the acupoints in the present study.

In the present study, memory loss was induced in rats by administering scopolamine. Scopolamine causes the loss of the ability to learn and form short-term memory in rodents and humans [18,19], with the hippocampus and frontal cortex being especially vulnerable [20]. Conditions that arise as a result of scopolamine administration include deterioration in the ability to perform complex exercises or motions, reduced motor intelligence, and impairment of the ability to start, plan, and assess tasks [21]. Because of these characteristics, scopamine-induced memory loss rat models have often been used in studies looking for an effective treatment for forgetfulness or dementia [22].

MWM is the measurement of an animal’s long-term memory [23]. Latency time in the MWM, in the HT8 + LR2 group showed that less time was spent in finding the escape platform as compared to the control group (Fig. 2). Where the number of target crossings in the HT8 + LR2 group were studied it was shown that the rats travelled more towards the previous location of the escape platform than the control group (Fig. 3). The time zone percentage for the LR2 and HT8 + LR2 groups showed that they stayed longer in the zone where the escape platform was located compared with the control group (Fig. 4). The T-Maze test measures an animal’s short-term memory [24] and the HT8 + LR2 group was shown to be more proficient at finding each arm in the correct order compared with the control group (Fig. 5). These findings indicated that HT8 and LR2 acupuncture was able to improve the learning ability and memory of rats, and it is believed that such treatments may be effective in improving memory and cognitive function in MCI patients.

The level of BDNF, a neurotrophic factor released by the brain, in patients with dementia is low [25]. Since BDNF plays an important role in regulation of neurotransmitters, and neuronal development and maintenance, increased BDNF activity may reduce cognitive impairment by facilitating neurotransmission. GPx and SOD are typical antioxidants that block reactive oxygen species (ROS) which are known to be the primary cause of various diseases including cancer and dementia by damaging the cell membrane and nucleus. GPx and SOD are antioxidative enzymes that help to inhibit ROS in the body to prevent tissue damage. In addition, they are known to affect systems that protect the brain by inhibiting oxidation to protect specific cell concentrations and tissues. Therefore, increased GPx and SOD activity may increase antioxidant activity which inhibits ROS, and such increased antioxidant activity may be effective in preventing cognitive impairment by protecting the brain through inhibition of ROS in the body [26-28].

ChAT is a key indicator of cholinergic neuronal function in the central and peripheral nervous systems. ChAT activity is significantly reduced in an aged brain and severity of cognitive impairment is known to be associated with ChAT inactivity [29]. Acetylcholine (ACh) is synthesized by ChAT, and it is one of the most important transmitters present in the brain tissue as a substance required for regulating various cognitive processes in the central and peripheral nervous systems. It is synthesized by the actions of acetyl Co-A and ChAT. ACh is degraded into acetate and choline by AChE. This enzyme, therefore, plays a role in terminating neurotransmission by ACh. Decreased levels of ACh released by synapses due to damage to cholinergic neurons, may cause learning and memory impairments [30]. It has been shown that enzymes involved in the synthesis and degradation of ACh include ChAT and AChE, and the neural activity of ChAT and AChE that affect ACh are very important for facilitation of neurotransmission in the body [31,32]. Moreover, patients with senile dementia, which accounts for more than 50% of all dementia cases, show a tendency towards decreased ChAT, and thus, methods which increase ACh levels in the body by inhibiting AChE are used to treat patients with dementia [33].

AChE neural activity was significantly lower in the HT8 + LR2 group than in the control group (Fig. 9), whereas ChAT neural activity was significantly higher in the HT8 + LR2 group than in the control group (Fig. 10). The level of neural activity was identified through the level of intensity of the staining in brain tissues (Figs. 11, 12). Therefore, it is believed that HT8 and LR2 acupuncture improves memory and cognitive impairment by inhibiting increased production of ACh and degradation through increased ChAT activity and inhibition of AChE activity in the hippocampus. Based on the results described in this study, it is believed that HT8 and LR2 acupuncture helps to improve behavior response time and improve memory in rats. These treatments also help to activate neurons by increasing the activity of BDNF, GPx, and SOD to protect the brain from ROS. They also reduce AChE neural activity, which inhibits degradation of ACh, and increase ChAT neural activity, which in turn increases ACh production.

In the present study, applying acupuncture on a single HT8 or LR2 acupoint or non-acupoint, such as the buttocks, demonstrated an improvement in cognitive function, as compared to the control group. However, the differences were not significant. The only result derived from using a single acupoint that showed a significant difference was from the L2 group in the time zone percentage of MWM. Therefore, acupuncture on a single HT8 or LR2 acupoint showed little therapeutic effect with no statistically significant difference in comparison to the control group. Combining both HT8 and LR2 acupuncture resulted in significant improvement in cognitive function in all experimental groups as compared to the control group. It is assumed that such significant improvement in cognitive function is related to the theory in KM of a combined acupoint method that uses more than one acupoint in each meridian. Oh et al [34] also reported that treatment using combined acupoints was more effective than using a single acupoint.

Based on these findings, it is believed that combined HT8 and LR2 acupuncture can improve learning and memory functions by enhancing cholinergic neurotransmission in the central nervous system. HT8 and LR2 acupuncture have also been shown to be useful for treating MCI and improving brain function. This was a small study using a rat model, therefore, more detailed and diverse studies are needed on MCI and neurotransmitters and this needs to be translated into a large clinical study. Studies on single and combined acupoints using Sobyu (HT8), Haenggan (LR2) and Baekhoe (GV20) are needed to compare their efficacies and examine their mechanism and stability, since a study by
Lee et al [35] reported that Baekhoe (GV20) was effective against scopolamine-induced cognitive impairment in rat models. In a modern aging society, dementia is considered a growing medical problem from a national perspective. Therefore, the prevention and treatment of MCI or forgetfulness, which is considered a precursor to dementia, are expected to be of great importance in the future.

**Conflicts of Interest**

The authors have no conflicts of interest to declare.

**References**


