CHAPTER VII
EFFECT OF LASER ACUPUNCTURE AT "HT7" ON MEMORY DEFICIT AND NEURODEGENERATION IN ANIMAL MODEL OF ALZHEIMER'S DISEASE

1. Introduction

To date, the burden of Alzheimer's disease has been continually increasing globally, particularly in developing countries, many of which lie in the Asia-Pacific region (Venketasubramanian *et al.*, 2011). AD, the most common age-related neurodegenerative disorder and dementia, markedly disturbs the quality of life of patients, families and caregivers (Banerjee *et al.*, 2003). The current therapeutic strategy is still limited. All therapeutic strategies can only slow down the progression of the disease. Most of the pharmacological regimens which used for treating AD are targeting at the improved cholinergic function. Although, they are very effective, the cost is expensive and numerous side effects are still presented (Katz *et al.*, 1999). Therefore, the complementary alternative has gained much attention.

Acupuncture, one of the most commonly used alternative medicine in Asian countries, has been long term used for treating dementia and for enhancing intelligence (Chen *et al.*, 2004). Several lines of evidence also clearly demonstrate the cognitive-enhancing effect of needle acupuncture at shenmen or HT7 (Lee *et al.*, 2011). However, needle acupuncture is an invasive technique, therefore it can elicit pain and increase risk of infection. Moreover, the effective of acupuncture treatment is varied depending on the physician skill. To avoid this disadvantage, the acupoint stimulation with the noninvasive tool such as laser beam has been implemented.In addition to the disadvantages of needle acupuncture mentioned earlier, most treatments are performed by the stimulation numerous acupoints simultaneously. No scientific evidence concerning the effect of the stimulation of single acupoint on the neurodegeneration and cognitive function is available until now. Therefore, the hypothesis whether the stimulation of single acupoint "HT7", an acupoint which is widely used for treating neurological disorders, with low level laser beam can protect against neurodegeneration and cognitive impairment as that observed in AD has been

raised. To elucidate this hypothesis, this study aimed to determine the effect of laser acupuncture at HT7 acupoint on the neuron density in hippocampus and on cognitive impairment in an animal model of AD induced by AF64A, a cholinotoxin. The possible underlying mechanism was also explored.

2. Materials and Methods

2.1 Animals

Young, 8-week-old, adult male Wistar rats were used as experimental animals. They were obtained from the National Laboratory Animal Center, Salaya. The weights of the animals on the first day of experiment were 180-220 grams. They were housed 6 per cage, maintained in a 12:12 light: dark cycle, and given ad-libitum access to food and water. The experiments were performed to minimize animal suffering, and the experimental protocols were approved by the Institutional Animal Care and Use Committee, Khon Kaen University, Thailand (AEKKU 41/2554). All treatments in this study were performed once daily between 8.00 a.m. and 5.00 p.m.

2.2 Chemicals and surgical procedures

Ethylcholine aziridinium (AF64A) was purchased from Sigma–Aldrich Co., USA. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 60 mg/kg BW. Then, AF64A (2 nmol/2 µl) was bilaterally infused via an intracerebroventricular (i.c.v.) route with a 30-gauge needle via burr holes that had been drilled through the skull into both the right and the left lateral ventricles according to the following stereotaxic coordinates: posterior 0.8 mm, lateral ±1.5 mm, and ventral (from dura) 3.6 mm. The rate of infusion was 1.0 µl/min. The needle was left in place for 5 minutes after infusion and then slowly withdrawn.

2.3 AF64A administration

AF64A was prepared as described previously (Hanin, 1996). Briefly, an aqueous solution of acetylethylcholine mustard HCl (Sigma, St. Louis, MO) was adjusted to pH 11.3 with NaOH. After the solution had been stirred for 30 minutes at room temperature, the pH was lowered to 7.4 with the gradual addition of HCl and was again stirred for 60 minutes. The amount of AF64A was then adjusted to 2 nmol/2 µl.Distilled water was processed in the same manner as the preparation of AF64A and was designated as artificial cerebrospinal fluid (ACSF).

2.4 Experimental Protocol

All rats were randomly assigned to 6 groups of 6 animals each as follows: In Group I, the control group, the rats received no treatment. In Group II, the vehicle group, the rats were administered artificial cerebrospinal fluid (ACSF) bilaterally via an intracerebroventricular route. In Group III, the vehicle+laser acupuncture group, the rats were administered artificial cerebrospinal fluid (ACSF) via an intracerebroventricular route and were subjected to laser acupuncture treatment bilaterally at HT7. In Group IV, the AF64A group, the rats received intracerebroventricular administration of AF64A, a cholinotoxin. In Group V, the AF64A+sham laser acupuncture group, the rats received intracerebroventricular administration of AF64A and laser acupuncture treatment at a non-acupoint. In Group VI, the AF64A+laser acupuncture group, the rats received intracerebroventricular administration of AF64A and laser acupuncture treatment at the HT7 acupoint.

The rats were treated with laser acupuncture once daily for 14 days after the administration of AF64A. Then, they were assessed for spatial memory by using the Morris water maze test at day 1, day 7 and day 14 after AF64A injection. At the end of experiment, they were sacrificed, and their brains were isolated to identify oxidative damage markers, including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). In addition, the activity of acetylcholinesterase (AChE), the neuron density and the expression of pERK1/2 in the hippocampus were also determined.

2.5 Laser acupuncture treatment protocol

Fifteen minutes before laser acupuncture treatments, all rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal injection) to minimize stress. Laser acupuncture treatment was performed once daily for 14 days. The rats were treated with a laser instrument that operated with a continuous violet or blue laser beam at a wavelength of 405 nm, an output power of 100 mW, and a spot diameter of 500 μm for 10 minutes (Litscher *et al.*, 2010; Wang *et al.*, 2011). Laser acupuncture was applied in the rats either at the HT7 point (the transverse crease of the wrist of the forepaw, radial to the tendon of the muscle flexor carpi ulnaris) or at a point 2–4 mm lateral to the HT7 acupoint (Yoon *et al.*, 2010).

2.6 Determination of cognitive function

Cognitive function was evaluated using the Morris water maze test. The water maze consisted of a metal pool (170 cm in diameter × 58 cm high) filled with tap water (25 ºC, 40 cm deep). The pool was divided into 4 quadrants (Northeast, Southeast, Southwest, and Northwest). The water surface was covered with non-toxic milk. A removable platform was immersed below the water's surface at the center of one quadrant. For each animal, the location of the invisible platform was placed at the center of one quadrant and was kept at that location throughout training. The time that each animal spent to find and climb onto the hidden platform was recorded as the escape latency. In order to determine the capability of the animals to retrieve and retain information, the platform was removed 24 hr later, and the rats were released into the quadrant diagonally opposite the quadrant that contained the platform. The time that each animal spent in the region that had previously contained the platform was recorded as the retention time (Morris, 1984).

2.7 Determination of density of survival neuron in the hippocampus

2.7.1 Histological procedure

Following anesthesia with sodium pentobarbital (60 mg/kg BW), the brain fixation was carried out by transcardial perfusion with a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. After the perfusion, the brains were removed and stored overnight in a fixative solution that is used for perfusion. They were then infiltrated with 30% sucrose solution at and kept at 4° C. The specimens were frozen rapidly and the coronal sections at 10 μM thick were prepared using cryostat. All sections were rinsed in the phosphate buffer and picked up on slides coated with 0.01 % aqueous solution of a high molecular weight poly L-lysine.

2.7.2 Morphological analysis

Five coronal sections of each rat in each group were studied quantitatively. Neuronal countsin hippocampus were performed by eye using a 40x magnification with final field 255 μm^2. The observer was blind to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give total number of neurons per 255 μm^2. All data are represented as number of neurons per 255 μm^2.

2.8 Determination of oxidative stress markers and acetylcholinesterase (AChE) activity

Rats were perfused with cold saline solution to remove the blood from the brain tissue; then, the hippocampi were rapidly removed and stored at 80°C until used. To determine the oxidative stress markers and the AChE activity, we prepared the brains as homogenates, and we determined the MDA level by using the thiobarbituric acid reaction whereas we determined the GSH-Px, CAT, and SOD activities by using a spectrophotometric method(Ellman *et al.*, 1961; Eyer and Podhradsky, 1986; Goldblith and Proctor, 1950; McCord and Fridovich, 1969).

2.9 Western blot analysis

The hippocampus were removed and rapidly frozen at -80°C. The frozen tissues were homogenized in ice cold RIPA buffer with protease inhibitors. The dissolved proteins were collected after centrifugation at 10,000 g for 30 min, and the supernatant was then collected. Protein concentrations were determined using NANO drop Spectrophotometers. Equal amounts of protein (35 µg) were separated by SDS-PAGE on 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). Lanes on each gel were also loaded with prestained protein markers to assess completeness of electrophoretic transfer. After electrophoretic transfer to nitrocellulose membrane, the blots were incubated in a blocking buffer (5% skim milk in Tris-buffer saline with 0.05% Tween-20) for 1 h at room temperature, and then incubated overnight with using one of these antibodies: phospho-ERK1/2 (1:1,000, Cell Signaling Cell Signaling Technology, Inc., Boston, MA, USA), total ERK1/2 (1:1,000, Cell Signaling Cell Signaling Technology,Inc., Boston, MA, USA). After several washing steps, membranes were incubated with HRP-linked secondary antibody (1:2,000) for 1 hr at room temperature and signalswere visualized by chemiluminescence using a ECL kit (Pierce, ThermoScientific). Images were acquired by ImageQuant LAS 4000, GE Healthcare. Band densities were quantified with ImageQuant TL (IQTL) software, GE healthcare (Gong *et al.*, 2011).

2.10 Statistical analysis

Data were expressed as means ± S.E.M. and were analyzed statistically by using the one-way ANOVA test, followed by the post-hoc (LSD) test. The results were considered statistically significant for p-value < 0.05.

3. Results

3.1 Cognitive-enhancing effect of laser acupuncture

The effects of laser acupuncture on cognitive function are shown in Figs. 7-1 and 7-2. ACSF did not produce any significant changes in the escape latency and the retention time whereas rats treated with either AF64A or AF64A plus laser acupuncture at a sham acupoint showed an elevated escape latency, but a decreased retention time (p-value < 0.001 for all compared to the ACSF group), throughout the study period. The administration of laser acupuncture reversed the elevation of escape latency at 7 and 14 days after AF64A administration (p-value<0.05 and 0.001, respectively, compared to the AF64A-treated group) while no significant change was observed in rats treated with AF64A plus laser acupuncture at a sham acupoint. In addition, laser acupuncture at HT7 acupoint did not produce any significant change in rats that received ACSF.

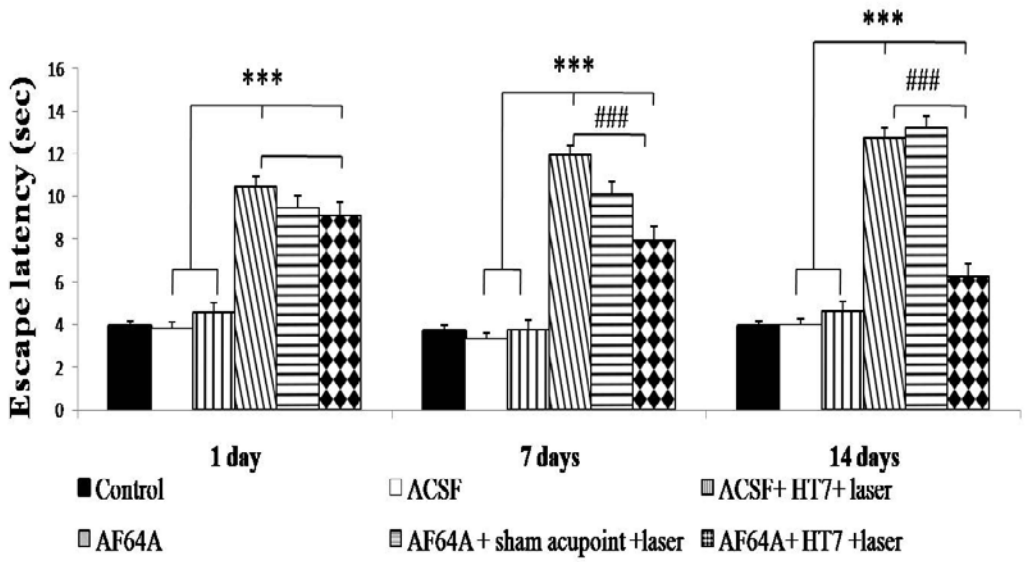

Figure 7-1 Effect of laser acupuncture on escape latency using the Morris water maze test in rats with Alzheimer's disease. Values given are the mean ± S.E.M. (n = 6). ***p-value< 0.001 as compared with the ACSF group and $^{###}$p-value < 0.001 as compared with the AF64A group

Figure 7-2 shows that rats that received AF64A showed reduced retention times at 7 and 14 days after AF64A administration (p-value<0.001 for all compared to the ACSF group). Laser acupuncture at the HT7 acupoint was found to be able to reverse the decreased retention time induced by AF64A (p-value < 0.01 and 0.001, respectively, compared to the AF64A-treated group). Again, no significant change in retention time was observed in rats that received ACSF plus laser acupuncture at HT7 acupoint.

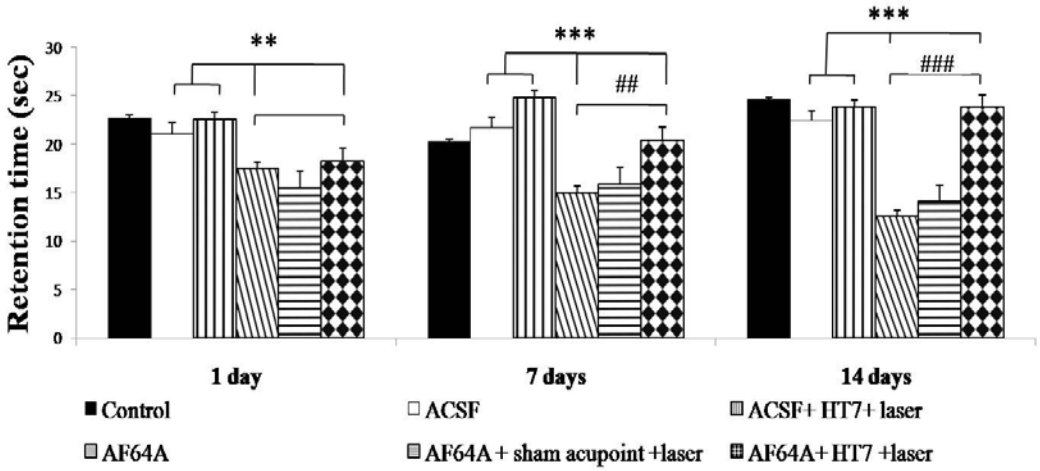

Figure 7-2 Effect of laser acupuncture on retention time using the Morris water maze test in rats with Alzheimer's disease. Values given are the mean ± S.E.M. (n = 6). **p-value<0.01, *** p-value<0.001 as compared with the ACSF group, and ## p-value<0.01, ###p-value<0.001 as compared with the AF64A group

3.2 Effect of laser acupuncture on hippocampal neurodegeneration

Figure 7-3 showed the effect of laser acupuncture on neuron density in hippocampus. The results showed that rats treated with either AF64A or AF64A plus laser acupuncture at a sham acupoint significantly decreased neurons density in CA1, CA2, CA3 and dentate gyrus (p-value<.001 all; compared to ACSF group). It was found that rats subjected to laser acupuncture at the HT7 acupoint significantly attenuated the reduction of neuron density in CA1, CA2 and CA3 (p-value<.01, .01 and .001 respectively; compared to the AF64A-treated group). Again, no significant change in neuron density was observed in rats that received ACSF plus laser acupuncture at HT7 acupoint.

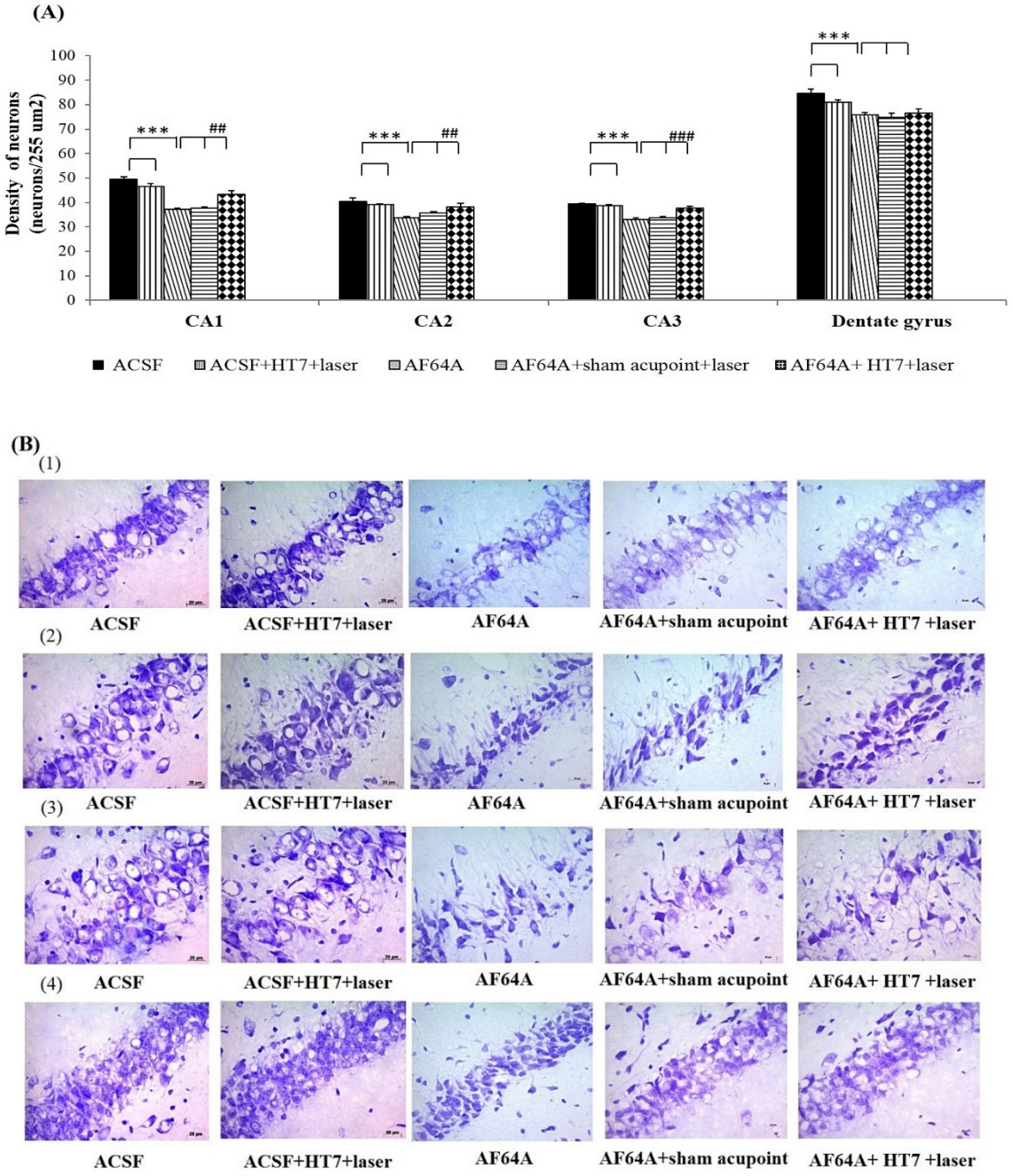

Figure 7-3 The effect of laser acupuncture on neuron density in various sub-regions of hippocampus including CA1,CA2,CA3 and dentate gyrus. A) Average density of neurons in CA1, CA2, CA3 and dentate gyrus B) Photograph of neuron density in CA1, CA2, CA3 and dentate gyrus. (n=6/group) *** p-value<.001; compared to ACSF group.##, ###p-value<.01 and .001 respectively; compared to AF64A group

3.3 Effect of laser acupuncture on oxidative stress markers and AChE enzyme activity

Figures 7-4 to 7-7 show the effects of laser acupuncture on the oxidative stress markers, including the MDA level and the activities of SOD, CAT and GSH-Px in the hippocampus. ACSF did not produce any significant changes in either the MDA level or the SOD, CAT and GSH-Px activities. Rats treated with AF64A had significantly enhanced MDA levels, but decreased CAT activities (p-value<0.01 and 0.001, respectively, compared to the ACSF group). Interestingly, rats that underwent laser acupuncture at the HT7 acupoint showed enhanced CAT and SOD activities, but no changes in GSH-Px activity and MDA level were observed.

The effect of laser acupuncture on the AChE activity in the hippocampus is shown in Fig. 7-8. ACSF produced no change in AChE activity. Rats treated with either AF64A or AF64A plus laser acupuncture at a sham acupoint showed elevated AChE activity in the hippocampus. However, this elevation wasattenuated by laser acupuncture at the HT7 acupoint.

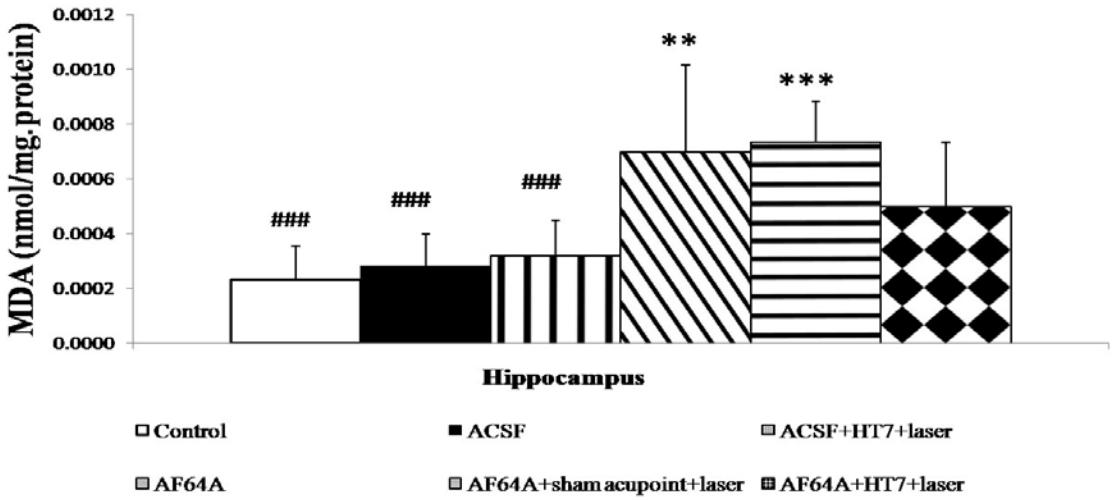

Figure 7-4 Effect of laser acupuncture on the level of malondialdehyde (MDA), a product of lipid peroxidation, in the hippocampus. Values given are the mean±S.E.M. (n = 6/group). **p-value<0.01, ***p-value<0.001 as compared with the ACSF group, and $^{###}$p-value < 0.001 as compared with the AF64A group

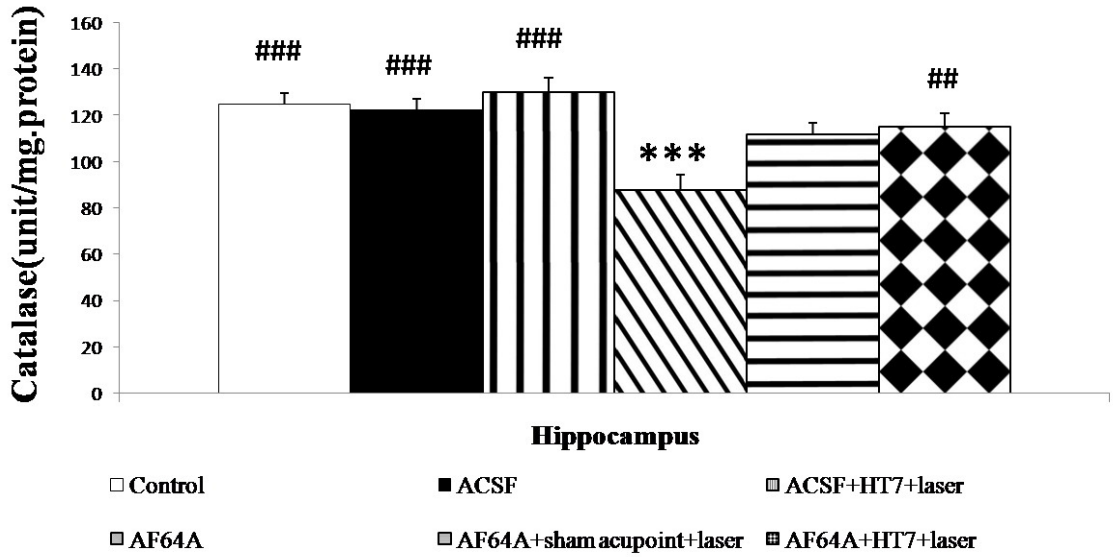

Figure 7-5 Effect of laser acupuncture on the activity of catalase (CAT) in the hippocampus. Values given are the mean ± S.E.M. (n = 6/group). *** p-value<0.001 as compared with the ACSF group, and $^{\#\#}$p-value<0.01, $^{\#\#\#}$p-value<0.001 as compared with the AF64A group

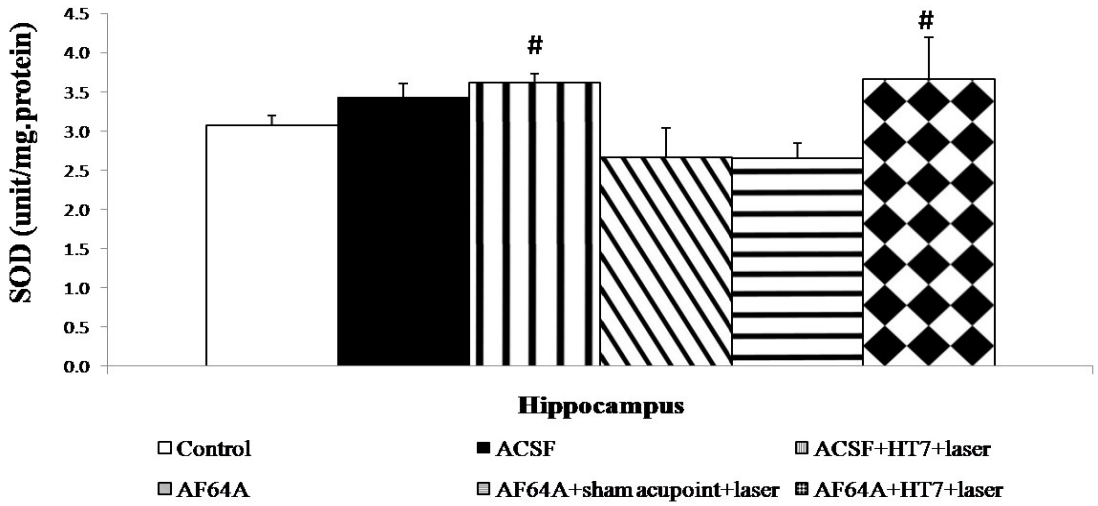

Figure 7-6 Effect of laser acupuncture on the activity of superoxide dismutase (SOD) in the hippocampus. Values given are the mean±S.E.M. (n= 6/ group). $^{\#}$ p-value < 0.05 as compared with the AF64A group

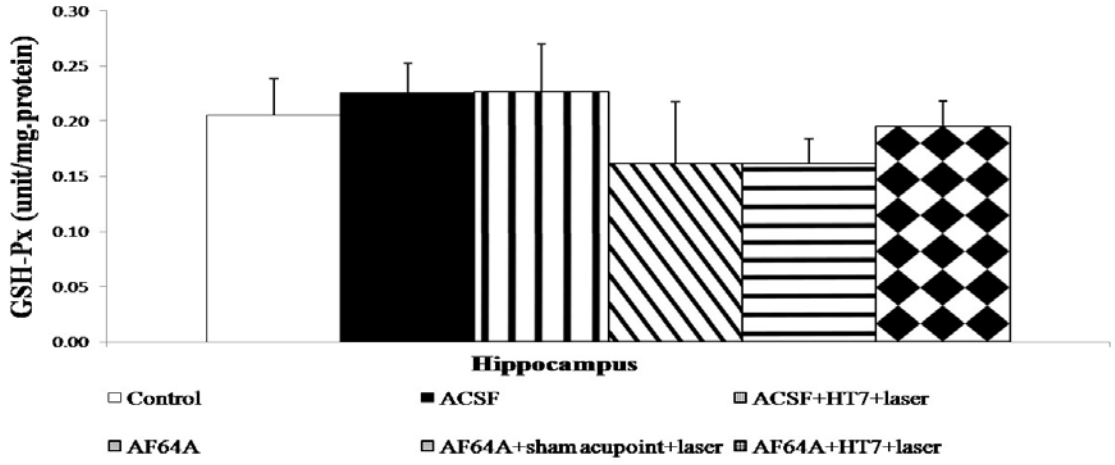

Figure 7-7 Effect of laser acupuncture on the activity of glutathione peroxidase (GSH-Px) in the hippocampus. Values given are the mean ± S.E.M. (n = 6/group)

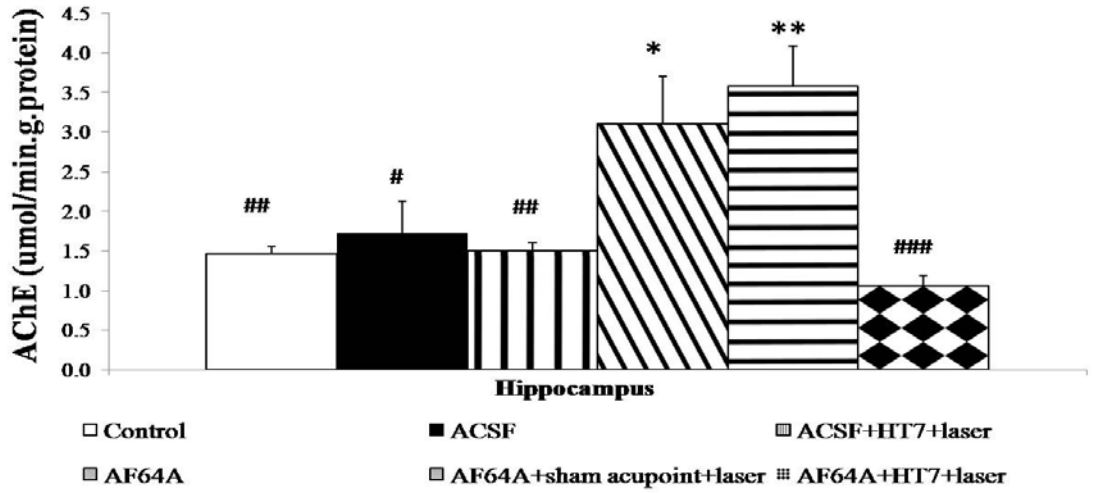

Figure 7-8 Effect of laser acupuncture on the activity of acetylcholinesterase (AChE) in the hippocampus. Values given are the mean ± S.E.M. (n = 6/group). *p-value<0.05, ** p-value<0.01 as compared with the ACSF group, and $^{\#}$ p-value<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$p-value<0.001 as compared with the AF64A group

3.4 Effect of laser acupuncture on ERK1/2 activation

The effect of laser acupuncture on ERK1/2 in the hippocampus was also assessed. The results were shown in figure 7-9. The results showed that laser acupuncture at HT7 acupoint in normal rats which received ACSF via intracerebroventricular route failed to show thee significant difference in pERK1/2 density in hippocampus. AF64A significantly decreased pERK1/2 density in hippocampus (p-value<.001; compared to ACSF treated group). Sham acupuncture failed to produce the significant change of pERK1/2 density in AF64A treated rats. Interestingly, laser acupuncture at HT7 acupoint significantly enhanced pERK1/2 density in hippocampus of AF64A treated rats (p-value<.05; compared to AF64A treated group).

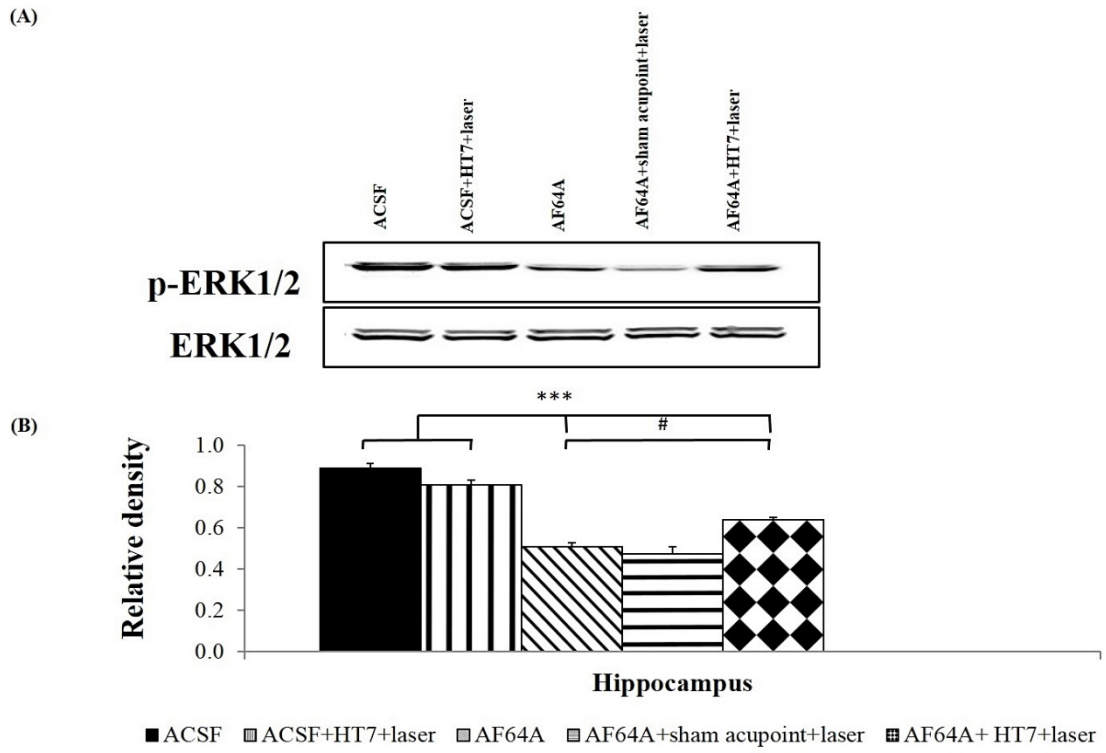

Figure 7-9 Effect of laser acupuncture on the level of ERK1/2 and pERK1/2 in hippocampus (n=6/group). ***p-value<.001; compared to ACSF group. $^{\#}$p-value<.05compared to AF64A group.

4. Discussion

This study has demonstrated the cognitive-enhancing effect of laser acupuncture and its positive modulation effect on oxidative stress and cholinergic function. In this study, a blue or violet laser beam at a wavelength of 405 nm was applied at the HT7 acupoint. Because the laser beam used in this study was a low-energy beam, this treatment is also referred to as low-level laser therapy (LLLT). LLLT is believed to produce photochemical, rather than thermal, effects because low irradiation levels are used and because no appreciable temperature rise takes place (Bjordal *et al.*, 2003; Kitchen and Partridge, 1991). LLLT may decrease the oxidative stress in neurons(Huang *et al.*, 2013). However, rats that received laser acupuncture at a sham acupointsshowed no changes in the MDA levels in their hippocampi. A possible explanation for the discrepancy between our study and the aforementioned study might be due to the differences in the selected brain areas and the types of exposure. This study was an in-vivo study whereas the previous study was an in-vitro study.

Laser acupuncture at HT7 significantly enhanced the CAT and the SOD activities without significant change in MDA level in the hippocampus. These data suggested that the restoration of oxidative stress balance might not play an important role in the cognitive-enhancing effect of laser acupuncture. In contrast to the MDA change, rats subjected to laser acupuncture mitigated the elevations of AChE activity, which in turn increased the available ACh in the hippocampus.

In addition to the suppression of AChE, the current results showed that laser acupuncture at HT7 significantly enhanced survival neuron and increased density of pERK1/2 bands. Since ERK cascade plays an important role in synaptic plasticity, long-term potentiation and cell survival, the increased ERK phosphorylation might contribute a role on the improved spatial learning and memory and the enhanced survival neuron (Giovannini, 2005; Gong *et al.*, 2011). Therefore, it is suggested that laser acupuncture at HT7 may improve cholinergic functionand neurodegeneration in hippocampus and resulted in the enhanced spatial memory capacity in an animal model of Alzheimer’s disease, as observed in this study.

In conclusion, this study is the first study to demonstrate the positive modulation effect of laser acupuncture at HT7 on the cholinergic function, which in

turn leads to reduced cognitive impairment in an animal model of Alzheimer's disease. Therefore, laser acupuncture at HT7 is a potential noninvasive strategy to attenuate memory impairment. Because laser acupuncture is very new and little information is available, detailed research, especially on the toxicity of repetitive exposure, is essential.

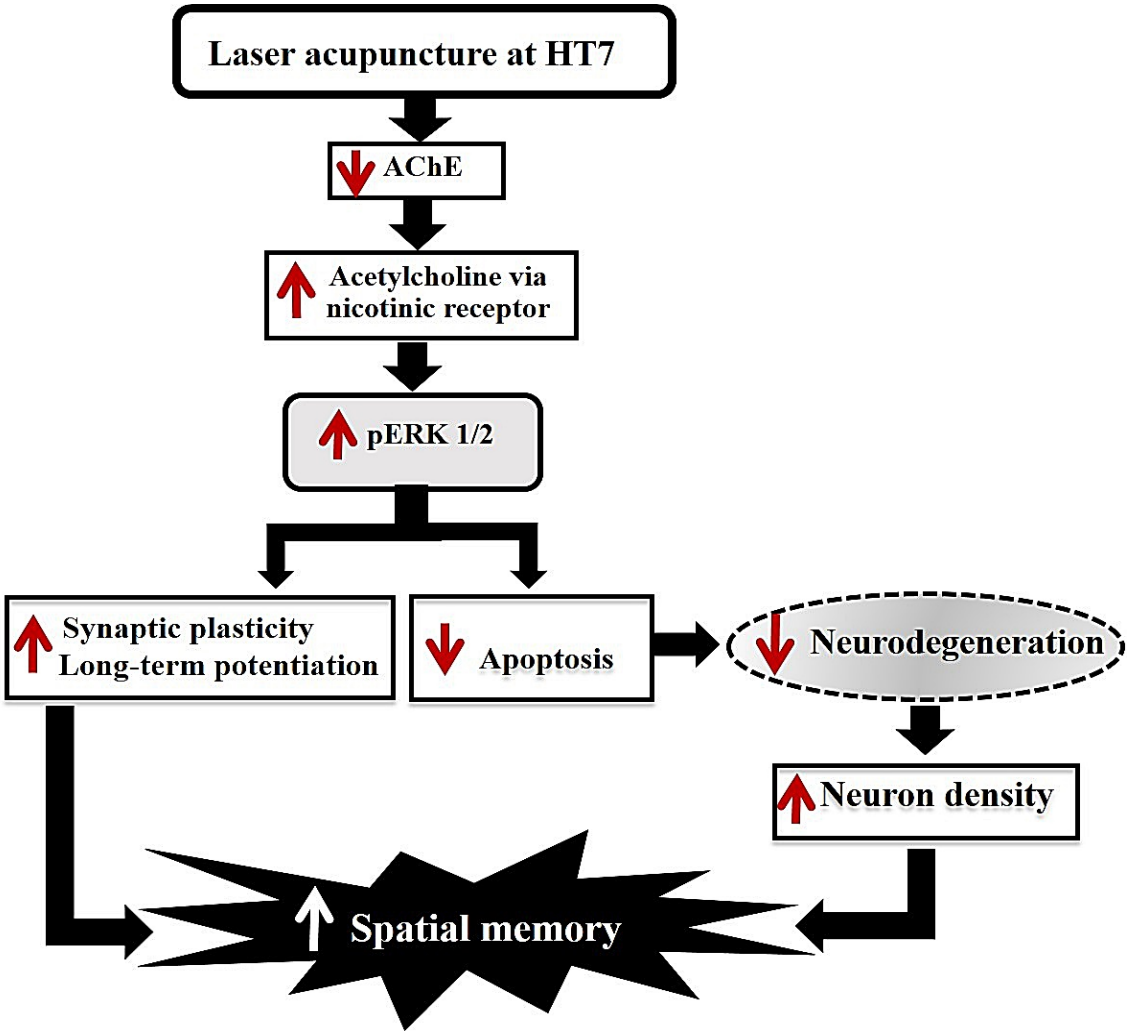

Figure 7-10 Schematic diagram concerning the possible mechanisms to improve spatial memory of laser acupuncture at HT7 in Alzheimer's disease rats induced by AF64A