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RESEARCH ARTICLE

Laser Acupuncture Improves Behavioral Disorders and Brain Oxidative Stress Status in the Valproic Acid Rat Model of Autism

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Abstract
The therapeutic strategy against autism, a severe neurological development disorder, is one of the challenges of this decade. Recent findings show that oxidative stress plays a crucial role on the pathophysiology of autism, and laser acupuncture at Shenmen (HT7) can improve oxidative status in many neurological disorders. Therefore, we aimed to assess the effect of laser acupuncture at HT7 on behavior disorders and oxidative stress status in the cortex, striatum, and hippocampus of the valproic acid rat model of autism. Laser acupuncture was performed once daily during postnatal day (PND) 14–PND 40. Behavioral tests including rotarod, open-field, learning and memory, and social behavior tests were performed during PND 14–PND 40. At the end of study, brain oxidative status including malondialdehyde levels and the activities of superoxide dismutase, catalase, and glutathione peroxidase were determined in the cortex, striatum, and hippocampus. Laser acupuncture at HT7 significantly improved autistic-like behaviors. Decreased malondialdehyde levels were observed in all areas mentioned above, however, increased glutathione peroxidase activity was observed only in the striatum and hippocampus. No changes in superoxide dismutase and catalase activities were observed in any investigated area of the brain. Therefore, our study suggests that laser acupuncture at HT7 partly mitigates autistic-like symptoms via improved oxidative status.

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1. Introduction

Autism, a severe and pervasive heterogeneous neurodevelopment disorder [1], is characterized by impaired social interaction and communication, repetitive behavioral patterns, and restricted interests [2]. The etiology of autism is still unclear, but it has been regarded as a multietiology disorder that is influenced by numerous factors, including genetic, environmental, and immunological factors, as well as oxidative stress [3]. Several lines of evidence have demonstrated that patients with autism show elevated levels of malondialdehyde (MDA) [4], a lipid peroxidation product, together with decreased levels of main scavenger enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) [5,6], in serum. Oxidative stress levels in the cortex and the cerebellar vermis are also elevated [7]. A proposal has been made that an elevation of oxidative stress in parts of the brain can impair or disturb brain development, resulting in the clinical manifestation of autism [4].

Recently, a randomized control trial study demonstrated that a 4-week electroacupuncture treatment at eight acupoints, Sisheencong (Ex-HN1), Yintang (EX-NH3), Neuguan (PC6), Shenmen (HT7), Taichong (LR3), Ear Naotean (AT3), Ear Shenmen (TF4), and Sanyinjiao (ST6), could improve many symptoms in autism spectrum disorder, including social initiation, receptive language, motor skills, coordination, and attention span [8]. Our previous work also clearly demonstrated that stimulation at HT7 could improve the status of oxidative stress in the brain and improve cognitive function in an animal model of Alzheimer's disease [9]. Based on this information and the crucial role of oxidative stress in autism pathophysiology, as mentioned earlier, laser acupuncture at HT7 has been thought to have a beneficial effect on various symptoms of autism. To the best of our knowledge, no scientific evidence concerning this issue has been available until now. Therefore, the current study aimed to determine the effect of laser acupuncture at HT7 on behavior disorders and on the status of oxidative stress in the cortex, striatum, and hippocampus of a valproic acid (VPA) rat model of autism.

2. Materials and methods

2.1. Animals

Pregnant female Wistar rats were obtained from the National Laboratory Animal Center, Salaya, Nakorn Pathom, Thailand. Rat pups, both male and female, were housed together in cages maintained in a 12-hour light:dark cycle and given *ad libitum* access to food and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee, Khon Kaen University, Thailand (AEKKU 56/2556).

2.2. Experimental protocol

Rat pups were randomly divided into four groups of 10 rats (5 females and 5 males) per group: control (naïve intact) group; VPA group; VPA plus laser acupuncture at HT7 group; and VPA + sham laser acupuncture. Rat pups not in the control group were treated with VPA and showed abnormal neurodevelopment. Laser acupuncture was performed once daily during postnatal days (PND) 14—40. Autism-like behaviors were observed using the rotarod, open-field, Morris water maze, and social behavior tests. On PND 41, the striatum, hippocampus, and cortex were isolated and oxidative stress markers including MDA level and the activities of SOD, CAT, and GSH-Px enzymes were assessed as shown in Fig. 1.

2.3. Autism-like condition induction

Rat pups at age 14 days with body weights of 18—30 g were injected with sodium valproate (Sigma Aldrich, St Louis, MO, USA) at a dose of 400 mg/kg body weight via a subcutaneous route. Autism-like symptoms were confirmed by using the reduced weight gain measured in weekly intervals, as well as the impaired olfactory discrimination on PND 9, delayed eye opening on PNDs 13 and 14, and the impaired motor development (swim performance) on PNDs 8 and 12 as initial activities reflecting abnormal neuron development in pups [10].

2.4. Laser acupuncture treatment

Five minutes prior to laser acupuncture treatment, rat pups were separated and housed individually. A blue laser beam (Xinland International Limited, Xi'an, Shaanxi, China) with a wavelength of 405 nm, a power output of 100 mW (0.100 J/second), and a diameter of 500 μm was continually administered at HT7 on both the left and the right sides. The blue laser treatment was performed once daily for 10 minutes from PND 14 to PND 40. In this study, the sham laser acupuncture group was treated with laser acupuncture at a location 2—4 mm lateral to HT7, as shown in Fig. 2.

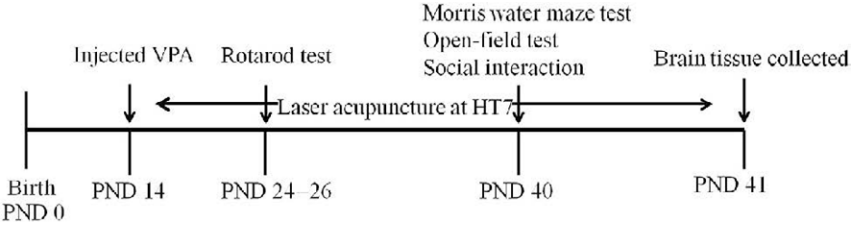

Figure 1 Schematic diagram for experimental protocol. PND = postnatal day; VPA = valproic acid.

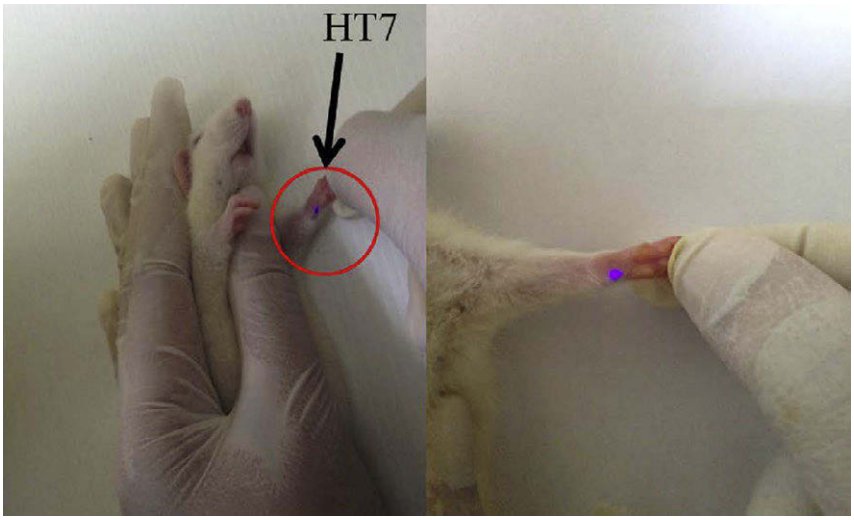

Figure 2 Photographs of location of laser acupuncture at HT7 acupoint.

2.5. Determination of behavior disorders

2.5.1. Rotarod test

Because children with autism often show difficulties in motor coordination and skill movements (motor learning) [11], we aimed to determine the effect of laser acupuncture on motor coordination by using the rotarod test during the period from PND 24 to PND 26. Rats were placed individually on the rotating rod. The rotating speed was gradually increased by 0.5 cm/second every 5 seconds, and the time taken by each animal to maintain its balance on the rod over a 5-minute period was recorded as the endurance time [12].

2.5.2. Morris water maze test

The effect of laser acupuncture on memory was evaluated based on the previous finding that autism patients often display a memory deficit [13]. The determination of spatial memory capacity via the water-maze test was performed on PND 40. The water maze consists of a metal pool (170 cm in diameter $\times$ 58 cm deep) filled with tap water (25°C, 40 cm deep) covered with nontoxic milk that was divided into four quadrants (northeast, southeast, southwest, and northwest). A removable platform was immersed below the water level at the center of one quadrant. The time that each animal spent trying to climb onto the immersed platform was recorded as the escape latency. Twenty-four hours later, the animal was re-exposed to the same condition, except that the immersed platform had been removed. Time spent in the region that had previously contained the platform was recorded as the retention time [14].

2.5.3. Open-field test

The open-field test was carried out to determine general locomotor activity and willingness to explore in rodents based on the information that children with autism show reduced environmental exploration [15]. On PND 40, the open-field test was performed. In brief, each animal was placed in the center of a large Plexiglas open-field chamber and allowed to explore the apparatus for 5 minutes. Grooming, rearing, and time spent on the periphery or in the center were assessed using a video tracking system [16].

2.5.4. Social interaction

Because impairment of social interactions is recognized as an important clinical manifestation in autism, we evaluated the effect of laser acupuncture on social interactions. The animals were separated and housed individually the night before the experiment to enhance social interactions. On PND 40, two animals from the same group, but different litters and cages, were placed into the test cage under red light for 20 minutes. Pairs were tested in a randomized order for groups, and the animals did not differ by $>$15 g in body weight. The frequencies of pinning (one rat lies on its back as the other stands with two paws on top of the first rat), following, grooming each other, sniffing of any body part, and sniffing of the anogenital body parts were taken as indicators of social engagement [17].

2.6. Determination of oxidative stress markers

At the end of the study, all rats were sacrificed. The cortex, striatum, and hippocampus were isolated and prepared as homogenates for the determination of oxidative stress markers. The oxidative stress marker MDA was evaluated using a thiobarbituric acid-reacting substances assay [18]. CAT, SOD, and GSH-Px were determined using a spectrophotometric method [19].

2.7. Statistical analysis

Data were presented as the mean $\pm$ standard error of the mean. The statistical significance of the data was determined using one-way analysis of variance (ANOVA), followed by Duncan's test. Statistical significance was achieved for $p < 0.05$.

3. Results

3.1. Effect of laser acupuncture at HT7 on behavior disorders

Fig. 3 shows the effect of laser acupuncture at HT7 on endurance time in the rotarod test. On PND 24, both VPA and VPA plus sham laser acupuncture groups significantly decreased endurance time ($p < 0.01$ all; compared with control group). Significant changes were also observed on PND 25 ($p < 0.001$ and $p < 0.01$, respectively; compared with control group) and PND 26 ($p < 0.01$ and $p < 0.05$, respectively; compared with control group). No significant differences in this parameter were observed between the VPA and the VPA plus sham laser acupuncture groups. Laser acupuncture at HT7 mitigated the decreased endurance time induced by VPA during PND 24—PND 26 ($p < 0.05$, 0.01, and $p < 0.05$, respectively; compared with VPA group). However, significant changes were observed only on PND 24—PND 25 ($p < 0.05$ and $p < 0.01$, respectively; compared with VPA plus sham laser treated group) when compared with VPA treated rats that received sham acupuncture.

Locomotor and exploratory behaviors were assessed via open-field test and the results are shown in Fig. 4. Fig. 4A reveals that both VPA and VPA plus sham laser acupuncture significantly decreased time spent in the center area ($p < 0.001$ all; compared with control group) but increased the time spent in the peripheral area ($p < 0.01$ all; compared with control group). Laser acupuncture at HT7 significantly mitigated the reduction of time spent in the center area and the increased time spent in the peripheral area induced by VPA ($p < 0.05$ all; compared with VPA group and $p < 0.05$ all; compared with VPA plus sham laser acupuncture group). It was found that VPA did not induce the significant changes on the number of rearing and grooming as shown in Fig. 4B.

VPA and VPA plus sham laser acupuncture at HT7 groups significantly decreased retention time in the Morris water maze test ($p < 0.001$ all; compared with control group) but failed to produce a significant change of escape latency in this test. Laser acupuncture at HT7 significantly attenuated the decreased retention time in VPA treated rats ($p < 0.001$; compared with VPA group and $p < 0.001$; compared with VPA plus sham acupuncture group). No significant changes in escape latency were observed in VPA plus sham acupuncture and VPA plus laser acupuncture at HT7 when compared with VPA and control groups as shown in Fig. 5.

Induction of autism by VPA significantly decreased the frequencies of following, grooming, and sniffing ($p < 0.05$ all; compared with the control group). The VPA plus sham laser acupuncture group also showed the significant decrease in all parameters mentioned earlier ($p < 0.05$ all; compared with control group). Sham laser acupuncture failed to alleviate any of the reductions induced by VPA in the frequencies of social behaviors; however, laser acupuncture at HT7 significantly mitigated the decreases in the frequencies of following and grooming ($p < 0.05$ all; compared with VPA group and $p < 0.05$ all; compared with VPA plus sham), as shown in Fig. 6.

3.2. Effect of laser acupuncture at HT7 on oxidative stress markers

Fig. 7 demonstrates that VPA-treated rats significantly increased MDA levels in the striatum, hippocampus, and cerebral cortex, ($p < 0.001$, 0.05, and $p < 0.01$, respectively, compared with the control group). The VPA plus sham laser acupuncture group also increased MDA levels in those three areas ($p < 0.001$, $p < 0.01$, and $p < 0.01$, respectively; compared with control group). Sham laser acupuncture failed to produce any significant attenuation effect on the MDA levels in the three regions whereas laser acupuncture at HT7 significantly mitigated the

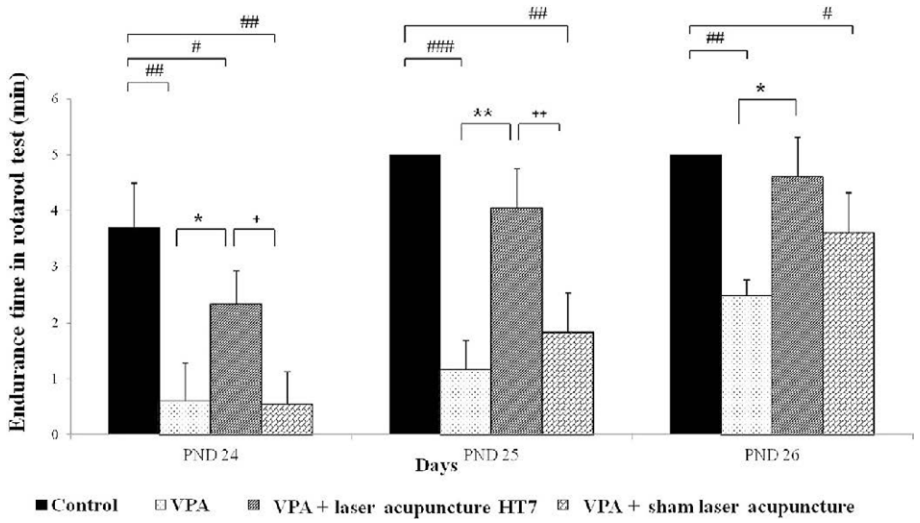

Figure 3 Effect of laser acupuncture at the HT7 acupoint on motor coordination in rats exposed to valproic acid (VPA). Values are expressed as mean ± standard error of the mean ($n = 10$). $^{\#, \#\#, \#\#\#}p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared with the control group. *, **$p < 0.05$ and $p < 0.01$, respectively, compared with the VPA group. $^{+, ++}p < 0.05$ and $p < 0.01$, respectively, compared with the VPA plus sham laser acupuncture group. PND = postnatal day.

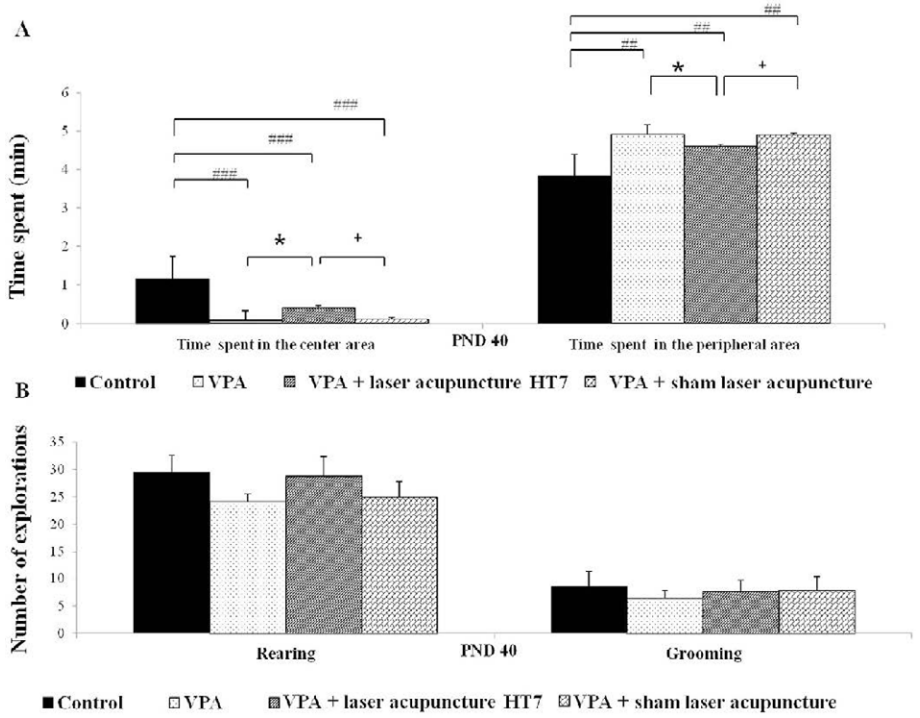

Figure 4 Effect of laser acupuncture at the HT7 acupoint on: (A) locomotor; and (B) exploratory activities in rats exposed to valproic acid (VPA). Values are expressed as mean ± standard error of the mean ($n = 10$). $^{\#\#}$, $^{\#\#\#}p < 0.01$ and $p < 0.001$, respectively, compared with the control group. $^{*}p < 0.05$ compared with the VPA group. $^{+}p < 0.05$ compared with the VPA plus sham laser acupuncture group. PND = postnatal day.

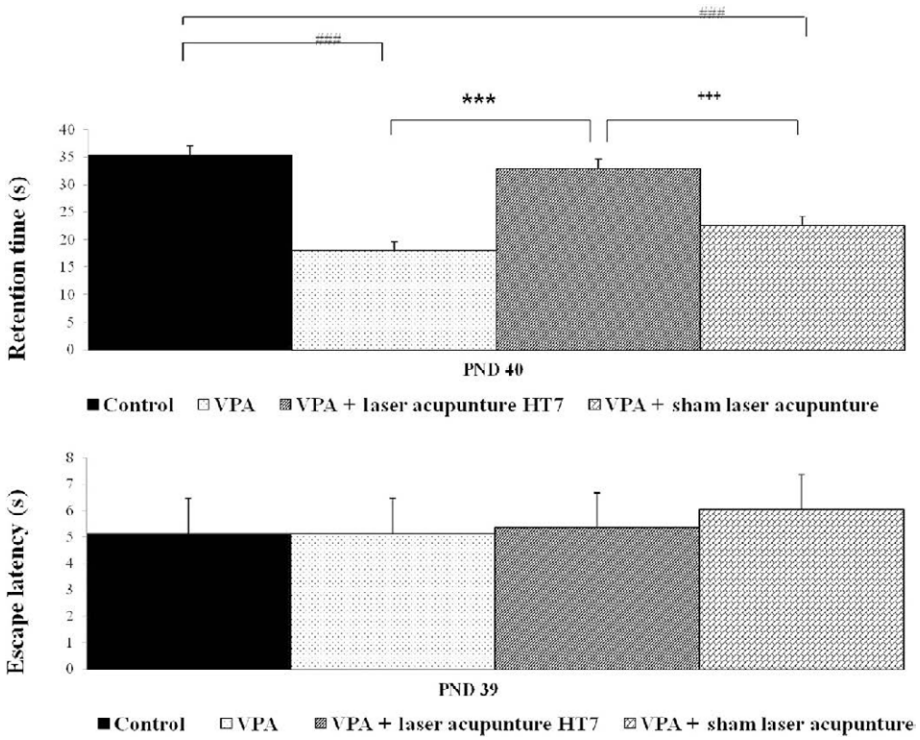

Figure 5 Effect of laser acupuncture at the HT7 acupoint on retention time and escape latency using the Morris water maze test in rats exposed to valproic acid (VPA). Values are expressed as mean ± standard error of the mean ($n = 10$). $^{\#\#\#}p < 0.001$ compared with the control group. $^{***}p < 0.001$ compared with the VPA group. $^{+++}p < 0.001$ compared with the VPA plus sham laser acupuncture group. PND = postnatal day.

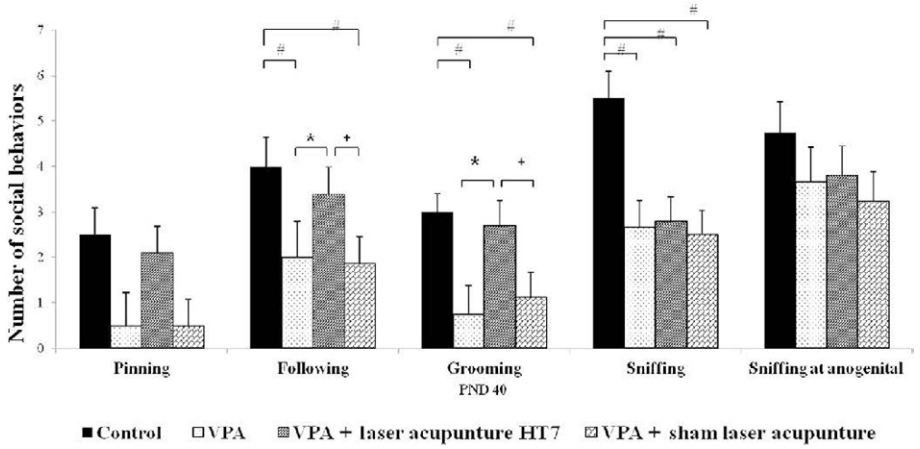

Figure 6 Effect of laser acupuncture at the HT7 acupoint on social behavior in rats exposed to valproic acid (VPA). Values are expressed as mean ± standard error of the mean ($n = 10$). $^{\#}p < 0.05$ compared with the control group. $^{*}p < 0.05$ compared with the VPA group. $^{+}p < 0.05$ compared with the VPA plus sham laser acupuncture group. PND = postnatal day.

elevations of the MDA levels in all areas investigated in this study ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively; compared with VPA group and $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively; compared with VPA plus sham acupuncture group). VPA administration significantly decreased the SOD activities in the striatum, hippocampus, and cerebral cortex ($p < 0.001$ all; compared with control group). Significant reductions in the GSH-Px activities were also observed in all areas mentioned earlier ($p < 0.001$, $p < 0.001$, and $p < 0.05$, respectively; compared with control group). However, decreased CAT activity was observed only in the hippocampus area ($p < 0.05$; compared with control group). Sham laser acupuncture did not significantly affect the above-mentioned changes in scavenger enzymes whereas laser acupuncture at HT7 significantly attenuated the decrease in the GSH-Px activity in both the striatum and the hippocampus ($p < 0.05$ and $p < 0.001$, respectively; compared with VPA plus sham acupuncture group and $p < 0.05$ all; compared with VPA plus sham acupuncture), as shown in Figs. 8—10.

4. Discussion

The development of various regions of mammal brains does not occur at a uniform pace. The timing of development varies among species and can occur both prenatally and postnatally. In rodent species, the development of most brain areas still persists during the postnatal period.

VPA was administered via a subcutaneous route on PND 9, the time when the rodent brain experiences a growth spurt [20] and when all developmental processes, including cell proliferation, synaptogenesis, elimination, and

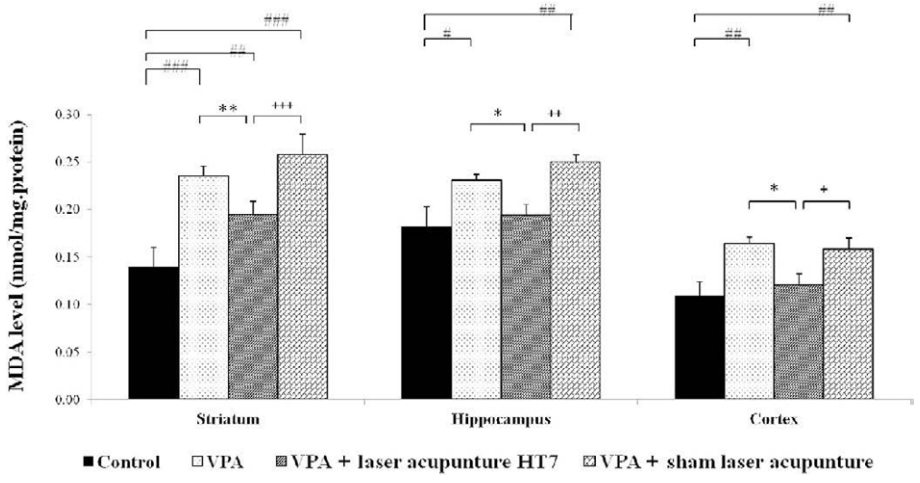

Figure 7 Effect of laser acupuncture at the HT7 acupoint on the level of malondialdehyde (MDA), a product of lipid peroxidation, in the striatum, hippocampus, and cortex. Values are expressed as mean ± standard error of the mean ($n = 10$). $^{\#, \#\#, \#\#\#}p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared with the control group. *, **$p < 0.05$ and $p < 0.01$, respectively, compared with the valproic acid (VPA) group. $^{+, ++, +++}p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared with the VPA plus sham laser acupuncture group.

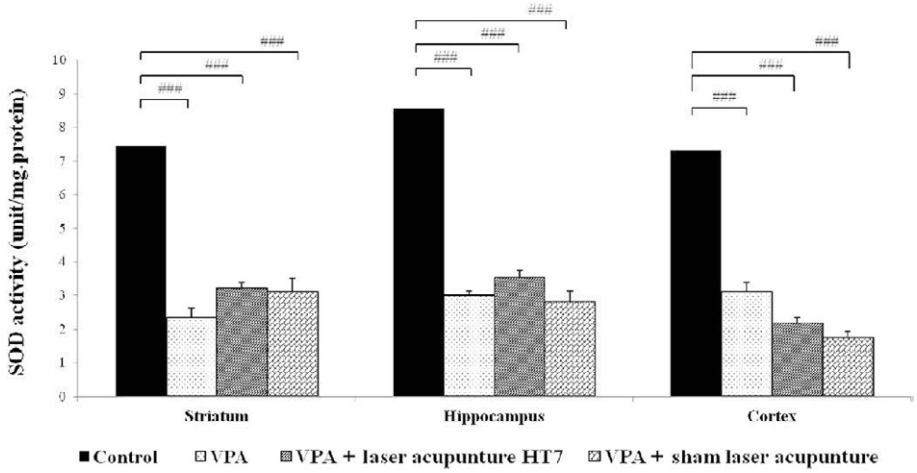

Figure 8 Effect of laser acupuncture at the HT7 acupoint on the activity of superoxide dismutase (SOD) in the striatum, hippocampus, and cortex. Values are expressed as mean ± standard error of the mean ($n = 10$). $^{###}p < 0.001$ compared with the control group.

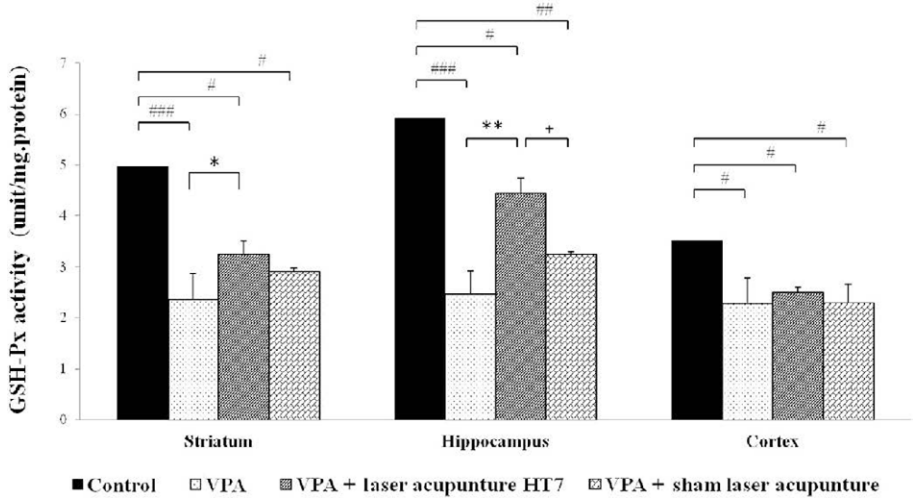

Figure 9 Effect of laser acupuncture at the HT7 acupoint on the activity of glutathione peroxidase (GSH-Px) in the striatum, hippocampus, and cortex. Values are expressed as mean ± standard error of the mean ($n = 10$). $^{\#, \#\#, \#\#\#}p<0.05$, 0.01 and 0.001, respectively, compared with the control group. *, **$p < 0.05$ and $p < 0.01$, respectively, compared with the valproic acid (VPA) group. $^{+}p < 0.05$ compared with the VPA plus sham laser acupuncture group.

myelination in most cortical and subcortical regions have not yet accomplished [21]. Therefore, the administered VPA might be absorbed and transported to the brain either via the choroid plexus (blood—cerebrospinal fluid barrier) or via the blood—brain barrier (blood capillary endothelium), and triggered the oxidative stress imbalance [22] giving rise to the damage of various brain structures such as the cerebral cortex [23]. This finding was also in agreement with our results demonstrating the elevation of MDA levels in the cerebral cortex. In addition, our study demonstrated that the elevation of oxidative stress occurred not only in the cerebral cortex but also in the striatum and the hippocampus. The elevation of oxidative stress can affect various cellular components, such as lipids, proteins, and DNA, and can affect metabolism and cellular activity up to neurodegeneration. Based on the crucial roles of the striatum in voluntary control [24] and in learning and memory [25], the roles of the cortex in motor, sensory, and social cognition [26], and the roles of both the cortex and the hippocampus in learning [27], we suggest that the abnormal motor behaviors might involve the elevation of oxidative stress in the cortex and the striatum whereas the impairment of learning might be associated with elevations of oxidative stress in the hippocampus. The elevation of oxidative stress in the cortex might also be involved in anxiety [28], and social recognition [26]. Our results also demonstrate that VPA-treated rats showed elevated MDA levels in the cerebral cortex, striatum, and hippocampus, together with decreased SOD and GSH-Px activities in all investigated areas whereas decreased CAT activity was observed only in the hippocampus. Therefore, the decreased scavenger enzyme activities in the

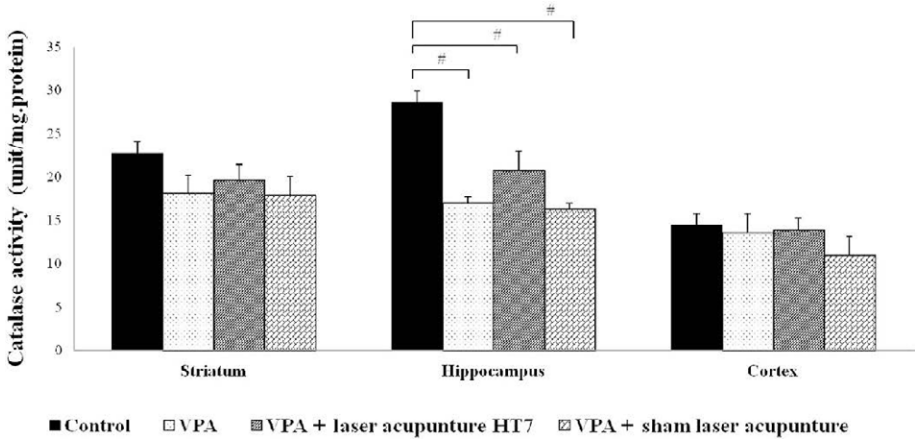

Figure 10 Effect of laser acupuncture at the HT7 acupoint on the activity of catalase in the striatum, hippocampus, and cortex. Values are expressed as mean ± standard error of the mean ($n = 10$). $^{\#}p < 0.05$ compared with the control group.

mentioned areas might be responsible for the elevated MDA levels.

In the VPA-treated rats, sham laser acupuncture failed to have any positive effects on any of the changes induced by VPA. However, laser acupuncture treated rats did reduce the enhanced MDA level induced by VPA. This suggests that the positive effect observed in this study might require specific laser stimulation at the HT7 acupoint. VPA-treated rats that received laser stimulation at the HT7 acupoint showed a decreased MDA level in the cerebral cortex without any changes of the scavenger enzymes. Therefore, the reduction in the MDA levels in the striatum and the hippocampus might be due the enhanced scavenger enzymes, especially SOD and GSH-Px. Based on the lack of any close relationship between the MDA level and the activities of scavenger enzymes in the cerebral cortex, the decreased MDA level in this area might be due to other factors such as decreased generation of oxidative stress, however, this requires further investigation.

In an animal model of autism, laser acupuncture at the HT7 acupoint is a potential, noninvasive strategy for repairing brain damage and reducing autism-like behaviors. This effect is partly due to decreased oxidative stress in the cortex, striatum, and hippocampus; thus this treatment strategy has an advantage for treating autism. However, further research concerning the effect of treatment duration and the precise underlying mechanism, as well as clinical trial studies, is necessary.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material of this manuscript.

Acknowledgments

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