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Laser Acupuncture at HT7 Improves the Cerebellar Disorders in Valproic Acid-Rat Model of Autism

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Abstract

The novel therapeutic strategy against autism is essential due to the limited therapeutic efficacy. Based on the benefit of laser acupuncture at HT7 acupoint on the neurological disorders related with oxidative stress and inflammation, its benefit on oxidative stress, neuroinflammation, and GABAergic/glutamatergic imbalance in cerebellum of autism have been considered. To elucidate this issue, male rat pups were induced autistic-like conditions by valproic acid (VPA) and treated with laser acupuncture at HT7 acupoint once daily between postnatal Day 14 and Day 40. At the end of study, the changes of oxidative stress markers, the expressions of cytokines interleukin 6 (IL-6) and glutamic acid decarboxylase (GAD) proteins (65 kDa and 67 kDa) together with gamma-aminobutyric acid transaminase (GABA-T) activity and density of Purkinje cell in the cerebellum were assessed. The results showed that laser acupuncture HT7 decreased oxidative stress, IL-6 expression, and GABA-T activity but increased the expressions of GAD 65 kDa together with the density of Purkinje cells in the cerebellum. Therefore, laser acupuncture at HT7 is the potential strategy to improve the cerebellar disorders in VPA-rat model of autism. The mechanism may occur partly via the decrease of oxidative stress status, inflammation, and the improved GABAergic function.

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1. Introduction

In recent decades, numerous lines of evidence point out that the cerebellum is one of the key brain regions affected in autism [1] and may serve as an important etiology of autism [2–5]. Data obtained from postmortem studies have revealed that most autistic patients show cerebellar hypoplasia and the decreased number of Purkinje cells [6–8]. In addition to the structural changes of the cerebellum, the reduction of the GABAergic function [9,10] and the increased oxidative stress status [11,12] in the cerebellum are also reported. It has been suggested that the cerebellar disorders are linked to sensorimotor, language, and social interaction deficits in autism [13].

Despite the advancement of technology, no therapeutic strategy can cure autism. Most current therapeutic strategies focus on the symptomatic treatment. Therefore, the novel effective therapeutic strategy is still required. Accumulative lines of evidence have demonstrated that laser acupuncture, an alternative stimulation acupoint with laser light, can improve many neurological disorders such as laser acupuncture, an alternative stimulation acupoint with laser light, can improve many neurological disorders such as autism spectrum disorder, and Parkinson’s disease [15]. In addition, our previous work has demonstrated that laser acupuncture at HT7 acupoint can mitigate autistic-like symptoms partly via improved oxidative status [16]. As the cerebellum has been proposed to play an important role on the pathophysiology of autism, it is interesting to understand the effect of laser acupuncture at the HT7 acupoint on both structural and functional changes of the cerebellum. Therefore, this study aimed to determine the effect of laser acupuncture at the HT7 acupoint on the structural and biochemical changes of the cerebellum in valproic acid-rat model of autism. The possible underlying mechanism was also investigated.

2. Materials and methods

2.1. Animals and experimental protocol

All experimental protocols used in this study had been approved by the Institutional Animal Care and Use Committee Khon Kaen University, Khon Kaen, Thailand (AEKKU 56/2556). On postnatal Day 14, the experimental animals were housed together in a cage, maintained in a 12:12 hour light–dark cycle, and given ad libitum access to food and water.

2.2. Experimental protocol

Male rat pups (18–30 g) were induced with autistic-like symptoms by valproic acid or VPA (Sigma-Aldrich, USA) at a dose of 400 mg/kg BW via intraperitoneal injection on postnatal Day (PND) 14 [16,17]. Then, the experimental animals were divided into four groups (n = 6) as following: (1) Group I, control group: rat pups received no treatment; (2) Group II, VPA group: rat pups were injected VPA alone; (3) Group III, VPA + sham laser acupuncture group: rat pups were injected VPA and received laser acupuncture treatments at a point 2–4 mm lateral to HT7; and (4) Group IV, VPA + laser acupuncture HT7 group: rat pups were injected VPA and received acupuncture treatment at the HT7 acupoint bilaterally.

Between PND14 and PND40, rats in Groups II–IV were subjected to the assigned treatments once daily. On PND 41, they were sacrificed and the cerebellum hemispheres were isolated for determining oxidative stress markers, including malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) enzymes. Moreover, Purkinje cell’s density, gamma aminobutyric acid transaminase (GABA-T) activity and the expressions of glutamate decarboxylase and interleukin-6 (IL-6) were also investigated. The schematic diagram showing the experimental protocols is presented in Fig. 1.

2.3. Laser acupuncture treatment

Five minutes prior to treatment, the rat pups were separated and housed individually. A continuous blue laser beam (Xinland International Limited, Hongguang Rd, Lianhu District, Xi’an, Shaanxi, China) with a wavelength of 405 nm, an output power of 100 mW (0.100 J/s), and a spot diameter of 500 μm was applied to each animal for 10 minutes once daily between PND14 and PND40. The animals in Group IV received laser application at HT7 acupoint (the transverse crease of the wrist of the forepaw, radial to the tendon of the muscle flexor carpi ulnaris) whereas those in Group III were exposed to a laser at a point 2–4 mm lateral to the HT7 acupoint [18].

2.4. Preparation of tissue homogenate and protein determination

After the last laser administration, the rat pup was anesthetized with intraperitoneal injection of sodium pentobarbital. Cerebellum hemisphere was isolated and kept cool and prepared as cerebellar homogenate. In brief, cerebellar hemisphere was homogenized in 4% of 0.2M PBS buffer pH 7.4 and subjected to a 12,000 rpm centrifugation at 4°C for 15 minutes. The supernatant was harvested and proteins determined by the method of Lowry [19] using bovine serum albumin as standard solution.

2.5. Determination of oxidative stress status

To determine the oxidative stress status, the determination of malondialdehyde (MDA) level and the activities of main scavenger enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were carried out.

The measurement of malondialdehyde (MDA), a lipid peroxidation process marker, was performed using the thiobarbituric acid reaction method. In brief, 750 μL of 20% acetic acid, 100 μL of 8.1% sodium dodecyl sulfate, and 750 μL of thiobarbituric acid were mixed with 100 μL of supernatant. Then, the mixture was boiled in a water bath at 95°C for 1 hour. After cooling to the room temperature, 500 μL of distilled water (DW) and 2500 μL of n-butanol and pyridine were added and centrifuged at 4000 rpm for 10 minutes. Absorbance was determined at wavelength 532 nm via spectrophotometer. MDA level was calculated using a series of calibration curves.
of standard solution of 1,1,3,3-tetramethoxypropane and expressed as nmol/mg protein [20].

SOD activity was assessed based on the reaction in a xanthine/xanthine oxidase system. In brief, 20 μL of supernatant and 20 μL of 5 units XOD were mixed with 200 μL of the solution containing 0.1M PBS pH 7.8, 10.7mM EDTA, 1.1mM cytochrom C and 0.108mM xanthine. The absorbance at 550 nm was measured via spectrophotometer and the activity of SOD was presented as units per milligram of protein [21].

Catalase activity was measured based on the rate of H₂O₂ decomposition. In brief, 10 μL of supernatant was mixed with 150 μL of 50mM potassium phosphate, 25 μL of 5N H₂SO₄, and 50 μL of 0.1M H₂O₂ at room temperature under dark conditions. Absorbance at 490 nm was read using a spectrophotometer. Activity was presented as units per milligram of protein [22].

GSH-Px activity was determined as previous described [16] based on the original method [23] with a slight modification. In brief, 10 μL of sample supernatant was mixed with the solution containing 10 μL of 48mM sodium phosphate, 0.02mM DL-dithiothreitol, 100 μL of 0.95mM sodium azide, 10 μL of 1mM glutathione, and 100 μL of 30% H₂O₂. Then, the mixed solution was shaken for 10 minutes before adding 10 μL of DTNB. The absorbance at 412 nm was recorded using a spectrophotometer and GSH-Px activity was presented as units per milligram of protein.

2.6. Determination of GABA transaminase activity

The cerebellum hemisphere was homogenized with cool buffer consisting of 0.5% Triton X-100, 5mM dithiothreitol, 1mM pyridoxal phosphate, and 10mM sodium phosphate buffer pH 7. The homogenate was centrifuged at 2000 rpm for 20 minutes at 0°C. The supernatant was harvested and used for the determination of GABA transaminase (GABA-T). In brief, 10 μL of supernatant was mixed with 40 μL of solution containing 20mM GABA, 10mM α-ketoglutarate, and 0.5mM NAD in 0.05M sodium phosphate buffer pH 8.0. After the 30-minute incubation at 30°C, the absorbance at 340 nm was recorded using a spectrophotometer. The enzyme activity was presented as mMol/milligram of protein [24].

2.7. Western blot analysis

The cerebellar hemisphere was homogenized in M-per reagent containing protease inhibitors (Thermo Fisher Scientific, Pierce Biotechnology, Inc., USA). The homogenate was subjected to a 20-minute centrifugation (10,000 rpm) at 4°C. Supernatant was collected for determining total protein using NANO drop spectrophotometer (NanoDrop ND-1000 Spectrophotometer V3.5 User’s Manual, NanoDrop Technologies Inc., USA). The supernatant protein was separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, USA). The membrane was incubated in a blocking buffer (5% skim milk in Tris-buffer saline with 0.05% Tween-20) for 1 hour at room temperature. After the blocking, the membrane was incubated with primary antibody for 2 hours at room temperature (1:5000 anti GAD65/67 or 1:2000 α-actin, Sigma-Aldrich) followed by a 1-hour incubation with secondary antibody (HRP conjugated antirabbit IgG 1:2000, Abcam, USA). The expression of GAD 65/67 was visualized by using the ECL plus substrate (Thermo Fisher Scientific, Pierce Biotechnology, Inc., USA).

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2.8. Immunohistochemical evaluation

Frozen sections of cerebellum hemisphere were cut 10 μm thick. The slides were washed with 0.1 M phosphate buffered saline (PBS) pH 7.4 for 5 minutes and exposed to high power (800 watts) microwave for 5 minutes. The blocking of endogenous peroxidase activity was performed by incubating sections with 0.5% H₂O₂ in methanol for 10 minutes. Then, the sections were incubated with mouse monoclonal IL-6 antibody (1:100, Abcam) at 4°C overnight followed by a 1-hour incubation with secondary biotinylated goat anti-mouse IgG antibody (1:2000, Abcam) at room temperature. The immunoperoxidase reaction product was visualized by using a 30-minute incubation at room temperature with DAKO kit (The DAKO EnVision+ System, Biocompare Inc., USA). The color was developed by incubating with 0.05% diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) for 5 minutes. Finally, all slides were dehydrated with ethanol and xylene before mounting. Density of IL-6 positive neuron was determined under light microscope (Olympus BH-2 BHS/BHT System Microscopes, 100× magnification) [26] by ImageJ program (Java Software, USA) [27].

2.9. Histological study

Cerebellar hemisphere was embedded in 4% paraformaldehyde and postfixed in 30% sucrose in phosphate buffer overnight. Serial of brain frozen sections at 10 μm thick were prepared via cryostat and stained with hematoxylin and eosin [28]. The evaluation of Purkinje density in cerebellum was performed under light microscope at 40× magnification. Counts were made in three adjacent fields and the mean number was calculated and expressed as density of neurons per 255 μm².

2.10. Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). All statistical analyses were carried out using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). The distributions of the variables were assessed using the Kolmogorov–Smirnov test. The one-way analysis of variance (ANOVA) with post hoc LSD test was performed to compare continuous variables. The statistical difference was regarded at p < 0.05.

3. Results

3.1. Oxidative stress status

Table 1 showed that VPA-rat pups increased MDA levels but decreased SOD, CAT, and GSH-Px activities in cerebellar hemisphere (p < 0.001, p < 0.001, p < 0.001, and p < 0.05, respectively, compared with the control group). Sham laser acupuncture failed to modify all parameters mentioned earlier. However, laser acupuncture at HT7 acupoint significantly attenuated the changes induced by VPA. It was found that VPA-rats which received laser acupuncture at HT7 acupoint decreased MDA levels but increased CAT activity in cerebellar hemisphere (p < 0.001 and p < 0.05, respectively, compared with the VPA group).

3.2. GABAergic system

The alteration of GABAergic system was determined by measuring the activity of GABA-T, a key enzyme on GABA degradation, and the expression of two isoforms of glutamic acid decarboxylase, a crucial enzyme for GABA synthesis. In Fig. 2, it was found that VPA-rat pups increased GABA-T activity (p < 0.01, compared with the control group). Sham laser acupuncture produced failed to produce a significant change of this parameter in VPA-rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA level (nmol/mg.protein)</th>
<th>SOD activity (unit/mg.protein)</th>
<th>CAT activity (unit/mg.protein)</th>
<th>GSH-Px activity (unit/mg.protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.143 ± 0.01</td>
<td>6.179 ± 0.88</td>
<td>24.95 ± 2.25</td>
<td>3.87 ± 0.25</td>
</tr>
<tr>
<td>VPA</td>
<td>0.297 ± 0.05*</td>
<td>1.509 ± 0.30*</td>
<td>12.02 ± 4.90*</td>
<td>2.87 ± 0.36*</td>
</tr>
<tr>
<td>VPA+ sham laser acupuncture</td>
<td>0.373 ± 0.04†</td>
<td>1.320 ± 0.81†</td>
<td>14.88 ± 5.90†</td>
<td>2.21 ± 0.12†</td>
</tr>
<tr>
<td>VPA+ laser acupuncture HT7</td>
<td>0.172 ± 0.05*†</td>
<td>2.409 ± 0.31†</td>
<td>20.62 ± 4.09*†</td>
<td>2.85 ± 0.52†</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SEM of oxidative stress status markers, n = 6/group, one-way ANOVA and post hoc test in comparing between group. *p < 0.05, compared with the VPA group. †p < 0.001; compared with the VPA group. ‡p < 0.001; compared with the control group. §p < 0.01; compared with the control group. ¶p < 0.05; compared with the control group. ANOVA = analysis of variance; CAT = catalase; GSH-Px = glutathione peroxidase; MDA = malondialdehyde; SEM = standard error of the mean; SOD = superoxide dismutase; VPA = valproic acid.

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However, this elevation was attenuated by laser acupuncture at the HT7 acupoint ($p < 0.05$, compared with VPA group).

Our results also showed that VPA-rat pups significantly decreased the expression of GAD 65 and GAD 67 kDa ($p < 0.01$ and $p < 0.001$, compared with the control group). Sham acupuncture failed to modify GAD 65 and GAD 67 expressions in cerebellar hemisphere of VPA rats. Interestingly, laser acupuncture at HT7 acupoint significantly enhanced the expression of GAD 65 kDa in VPA-rats ($p < 0.05$ compared with the VPA group). Unfortunately, no change in GAD 67 kDa expression was observed in VPA-rats as shown in Fig. 3.

### 3.3. Histological and immunohistological changes

The interleukin-6 (IL-6) expression in cerebellar hemisphere was also determined based on its role on immune and inflammatory responses [29] and its modulation effect on autism-like behaviors through the impairments of...
synaptic formation, dendritic spine development, and neuronal circuit balance in autism [30]. In Fig. 4, it was found that VPA rats enhanced IL-6 positive neuron density in cerebellar hemisphere (\( p < 0.001 \), compared with the control group). Interestingly, laser acupuncture at HT7 acupoint attenuated the elevation of IL-6 positive neuron density induced by VPA in this area (\( p < 0.05 \) compared with VPA group). No significant change of the mentioned parameter was observed in VPA rats which received sham laser acupuncture.

The effect of laser acupuncture at HT7 acupoint on Purkinje cell density in the cerebellum was also performed and results were shown in Fig. 5. The results showed that VPA decreased the density of Purkinje neuron (\( p < 0.001 \), compared with the control group). This change was counteracted only by laser acupuncture at HT7 acupoint (\( p < 0.01 \), compared with VPA group). No change was observed in VPA-rats with sham laser acupuncture.

4. Discussion

As Purkinje cell loss in cerebellum has been reported to be linked to autism-like behavior [31,32], cerebellar changes in the autism model has gained much attention. Our data showed that VPA-rat pups decreased antioxidant activities but increased MDA level in the cerebellum. These data are in agreement with a previous study [33] which shows that Purkinje cell is vulnerable to oxidative stress resulting in degeneration [34,35]. Therefore, the decreased Purkinje cell density in VPA-rat pups might be due to the decreased SOD, CAT, and GSH-Px activities leading to an excess oxidative stress which in turn enhanced the attack of oxidative stress at lipid component of membrane resulting in the elevated MDA level and Purkinje cell degeneration. Besides oxidative stress, inflammation especially IL-6 also partially plays a role on the pathogenesis of autism [36,37]. The current data also show that IL-6 expression increases in cerebellum of VPA-rat pups.
which is in agreement with the change that is observed in an autistic brain. Our study has clearly demonstrated that laser acupuncture at HT7 acupoint decreased IL-6 in cerebellum of VPA-rats. Although low level laser therapy has been previously shown to improve brain oxidative stress status [38] and inflammation [39,40], we found that the laser application at nonacupoint failed to show the improved oxidative stress status and inflammation. Therefore, the positive modulation effect of laser acupuncture at HT7 acupoint observed in this study might not due to the benefit of laser alone. However, the possible explanation of how laser acupuncture at HT7 acupoint can improve oxidative stress status and inflammation in cerebellum still requires further exploration. The improved oxidative stress status and inflammation induced by laser acupuncture at HT7 might be responsible partly for the enhanced Purkinje cells leading to the increased GABAergic function.

Purkinje cell axons are regarded as the sole output of the cerebellar cortex and all synapses on cerebellar or vestibular nuclei. Cerebellar nuclei in turn connect broadly to various regions of the brain including motor cortex, association, and paralimbic cortices which contribute the crucial roles on motor coordination and balance, cognitive, and emotional functions [41]. Therefore, the increased Purkinje cell survival in cerebellum induced by laser acupuncture at HT7 acupoint might contribute to the role on the improved autistic-like behavior [15].

5. Conclusion

The present study is the first study to demonstrate that laser acupuncture at HT7 acupoint improves oxidative stress status and inflammation which in turn improves...
Purkinje cell loss and the reduction of GABAergic function in cerebellum. Therefore, laser acupuncture at HT7 acupuncture point is the potential strategy to improve cerebellar damage in VPA-rats model of autism.

Disclosure statement

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

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