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Original Article

Effect of Acupuncture on 6-Hydroxydopamine-induced Nigrostriatal Dopaminergic Neuronal Cell Death in Rats

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Objectives : Acupuncture treatment has been clinically used for functional recovery in Parkinson's disease. In the present study, we investigated the effect of acupuncture at Zusanli (ST36) on nigrostriatal dopaminergic neuronal cell death in rats.

Methods : A Parkinson's disease model was induced by the unilateral injection of 6-hydroxydopamine (6-OHDA) into the striatum. Acupuncture treatment was performed at Zusanli (ST36) and at the hip, as a non-acupoint, once a day for 14 days. Two weeks after 6-OHDA injection, an apomorphine-induced rotational behavior test showed significant rotational asymmetry in rats with Parkinson's disease. Immunostaining for tyrosine hydroxylase demonstrated a dopaminergic neuronal loss in the substantia nigra and dopaminergic fiber loss in the striatum.

Results : Acupuncture at the ST36 acupoint significantly inhibited rotational asymmetry in rats with Parkinson's disease, and also protected against 6-OHDA-induced nigrostriatal dopaminergic neuronal loss. These effects of acupuncture were not observed for non-acupoint acupuncture.

Conclusions : The present study shows that acupuncture treatment, especially at the ST36 acupoint, can be used as a useful strategy for the treatment of Parkinson's disease.

Key Words: Paeonia radix, Parkinson' s disease, 1-Methyl-4-phenylpyridine, Apoptosis

Introduction

Parkinson's disease (PD) is induced by the degeneration and loss of neurons in the midbrain substantia nigra, where the neurotransmitter dopamine is produced. The clinical characteristics of Parkinson's disease are tremor at rest, an inability to initiate or complete movements, muscle rigidity, postural instability, and a lack of facial expression[1]. In animals, the intrastriatal injection with 6-hydroxydopamine (6-OHDA), a neurotoxin which selectively injures catecholaminergic neurons, causes the progressive loss of nigral dopaminergic neurons, resulting in widespread loss of dopamine throughout the primary projection of these neurons in the nigrostriatum[2, 3]. Moreover, depletion of striatal dopamine induced by 6-OHDA injection shows symptoms such as bradykinesia, sensorimotor neglect, aphagia, adipsia, akathisia, short-step locomotion, and postural abnormalities, which resemble Parkinson's disease in human[4-6].

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of the catecholamine neurotransmitters such as dopamine, epinephrine, and norepinephrine. More specifically, it converts L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), the rate-limiting step in the synthesis of dopamine[7]. Since

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TH is a rate-limiting enzyme for the biosynthesis of dopamine, TH activity is progressively decreased following the loss of DA neurons in the substantia nigra in the patients with Parkinson's disease[8-10]. Moreover, TH immunohistochemistry has widely been used as an important method of detecting the injury or death of dopaminergic fibers and cell bodies[11-14].

Acupuncture has been utilized as a clinical treatment for various diseases in Oriental medicine and has many advantages as a treatment compared to medication-based therapies: i.e., safety, efficacy, convenience, and freedom from side-effects. Acupuncture treatment has also been applied to treat Parkinson's disease. Clinical studies have shown that acupuncture alleviates the symptoms of Parkinson's disease, such as tremor, walking difficulties, physical slowness, pain, sleep, depression, and anxiety[15-17], and delays the progression of these symptoms[15]. Animal studies have shown that acupuncture alleviates behavioral abnormalities in rats with Parkinson's disease and elevates the mRNA levels of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) in the substantia nigra of rats administered medial forebrain bundle transection[18-20].

The Zusanli (ST36), near the knee joint of the hind limb 2 mm lateral to the anterior tubercle of the tibia, is one of the most effective acupuncture points for brain diseases, with a wide range of analgesic, spasmolytic, and homeostatic effects[21]. Experimental studies have shown that acupuncture at ST36 stimulates cell proliferation in the dentate gyrus under pathologic conditions, including ischemia and diabetes[22-23].

Acupuncture at ST36 also decreases neuronal cell death following hemorrhage and ischemia in animals, thus demonstrating the neuroprotective effect of the ST36 acupoint against brain damage[24-25].

In the present study, the protective effect of acupuncture on nigrostriatal dopaminergic neuronal loss was investigated in rats with 6-OHDA-induced Parkinson' s disease by TH immunohistochemisty.

Materials and methods

1. Animals and treatments

Adult male Sprague-Dawley rats weighing 210±10 g (9 weeks in age) were used in this experiment. The rats were housed under controlled temperature (20±2 ℃) and lighting (7 a.m. to 7 p.m.) conditions. Food and water were available ad libitum before and after surgery. The experimental procedures were performed in accordance with the animal care guidelines of the National Institute of Health (NIH) and the Korean Academy of Medical Sciences. Animals were randomly assigned into six groups (n = 5 in each group): the sham-operation (control) group, the sham-operation and non-acupoint-acupunctured group, the sham-operation and Zusanli-acupunctured group, the 6-OHDA-treated group, the 6-OHDA-treated and non-acupoint-acupunctured group, the 6-OHDA-treated and Zusanli-acupunctured group.

2. 6-OHDA injection into the striatum

Rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.; Sigma Chemical Co., St. Louis, MO, USA) and placed in a stereotaxic frame. Through a hole drilled in the skull, a 26-gauge needle was implanted into the striatum at the following coordinates: 2.6 mm lateral to midline, 0.7 mm anterior to coronal suture, depth 4.5 mm deep from the surface of the brain. 6-OHDA (20 μg at 4 μg/μl) containing 0.2 mg/ml L-ascorbic acid was injected at the rate 1μl/min. The needle were left in place for an additional 5 min following the infusion, and then was slowly withdrawn. Animals in the sham operation groups were injected with an equivalent dose of physiological saline using the same method.

3. Acupuncture treatments

For acupuncture stimulation, stainless acupuncture needles of 0.3 mm diameter were bilaterally inserted about 2~4 mm depth into the locus of the Zusanli (ST36), located 5 mm lateral and distal to the anterior tubercle of the tibia for the Zusanli-acupunctured group and into the both side of hips for the non-acupoint-acupunctured group, and left in place for 20 min as a previously described method[22]. Acupuncture treatment was given to each animal once a day (10 a.m. or 5 p.m.) for 14 consecutive days, starting on the first day of the experiment.

4. Assessment of rotational behavior

Two weeks after the unilateral injection of 6-OHDA into the striatum, changes in rotational behavior induced by apomorphine (0.5 mg/kg, s.c.) were assessed using an automatic rotometer over 60 min period as previous described[26]. The net number of rotations was counted as follows: the number of contralateral rotations - the number of ipsilateral rotations.

5. Tissue preparation

Immediately after rotational behavior testing, animals were deeply anesthetized with Zoletil 50 (10mg/kg, i.p.; Vibac Laboratories, Carros, France), transcardially perfused with 50 mM phosphate- buffered saline (PBS) and fixed with 4% paraformaldehyde in 100 mM phosphate buffer (PB) at pH 7.4. The brain was removed, fixed in the fixative overnight, and transferred to 30% sucrose solution for cryoprotection. Serial coronal sections of 40 μm thick were made with a freezing microtome (Leica, Nussloch, Germany).

6. Immunohistochemistry for Tyrosine hydroxylase expression

Every fourth section in the substantia nigra was selected from each brain in the region spanning from Bregma -5.2 mm to -5.6 mm, and processed for TH-immunohistochemistry. The staining was carried out using free-floating sections. Sections were rinsed in PBS and incubated in 3% H2O2 for 20 min to block the endogenous peroxidase activity. After washing in PBS, the sections were incubated in blocking serum (10% normal horse serum and 0.1% Triton X-100 in PBS) for 30 min, followed by incubation in anti-TH mouse monoclonal antibody solution (1:1000, Chemicon, Temcula, USA) for 24 h at room temperature. The sections were then incubated for 1 h in biotinylated anti-mouse IgG secondary antibody 1:300 (Vector Laboratories, Burlingame, CA, USA). The sections were subsequently incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 1 h at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.05% 3,3-diaminobenzidine (DAB) and 0.01% H_2O_2 in 50 mM Tris buffer (pH 7.6) for 3 min. Sections were mounted on gelatine-coated slides and coverslipped with mounting medium.

The number of TH-immunoreactive neurons in the substantia nigra was counted in each section using a bright-field microscope (Olympus, Tokyo, Japan) and analyzed using an Image-Pro®Plus image analyzer (Media Cybernetics Inc., Silver Spring, MD, USA). The numbers of TH-positive cells in both sides of the substantia nigra were counted in four sections across the center of the mesencephalon to determine the TH-positive cell percentage in the lesion side versus the intact side. The survival rate of TH-positive cells in the substantia nigra was calculated as follows: the number of TH-positive cells in the lesion side/the number of TH-positive cells on the intact side × 100.

For TH-immunohistochemistry in the striatum, sections in the Bregma -0.7 mm were selected to quantify the optical densities of TH-immunoreactive fibers. TH-immunoreactive fiber density was measured

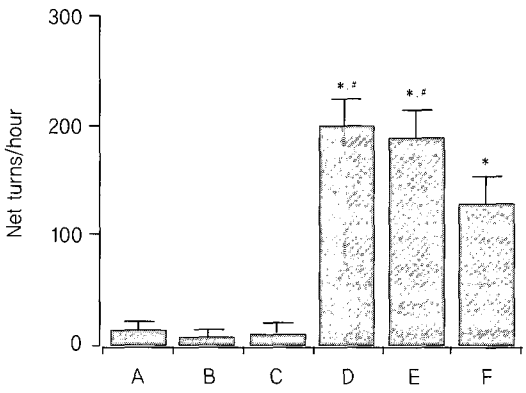

Fig. 1. Effect of acupuncture at ST36 on apomorphine-induced rotation in 6-OHDA-treated rats. Values are represented as the mean±S.E.M.. * represents $p < 0.05$ compared to the sham-operation groups. # represents $p < 0.05$ compared to the 6-OHDA-treated and Zusanli-acupuntured group. (A) Sham-operation group, (B) sham-operation and non-acupoint-acupunctured group, (C) sham-operation and Zusanli-acupunctured group, (D) 6-OHDA-treated group, (E) 6-OHDA-treated and non-acupoint-acupunctured group, (F) 6-OHDA-treated and Zusanli-acupunctured group.

in 100 × 100 μm square images of the dorsolateral striatum using an image analyzer (Multiscan, Fullerton, CA, USA). Images were acquired at 200 X using an image analyzer (Media Cybernetics Inc.). To estimate the TH-staining density, the optical densities were corrected for nonspecific background density, which was measured in completely denervated parts of the striatum. TH-positive fiber density ratios in the striatum were calculated as follows: optical density in the lesion side/optical density in the intact side.

7. Data analysis

All values were expressed as mean±standard error mean (S.E.M.). For comparisons among the groups, one-way analysis of variance (ANOVA) and Duncan's post-hoc test were performed with $p < 0.05$ as an indication of statistical significance.

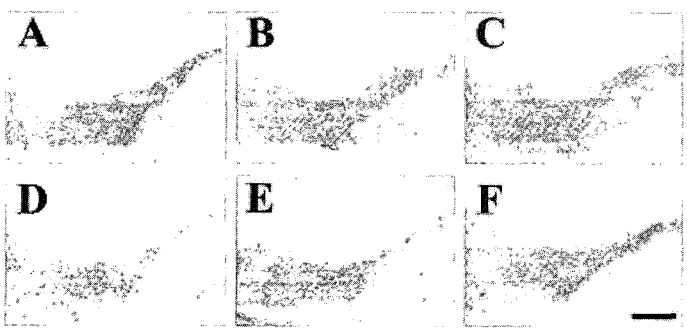

Fig. 2-1. Effect of acupuncture on the survival rate of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra in 6-OHDA-treated rats. Photomicrographs showing TH-specific immunohistochemical staining in the substantia nigra. The scale bar represents 200 μm. (A) Sham-operation group, (B) sham-operation and non-acupoint-acupunctured group, (C) sham-operation and Zusanli-acupunctured group, (D) 6-OHDA-treated group, (E) 6-OHDA-treated and non-acupoint-acupunctured group, (F) 6-OHDA-treated and Zusanli-acupunctured group.

Results

1. Effect of acupuncture at ST36 on apomorphine- induced rotation in 6-OHDA-treated rats

Fig. 1 shows the results from the assessment of apomorphine-induced changes in rotational behavior 2 weeks after injecting 6-OHDA into the striatum. The net number of rotations was calculated as the difference between the number of contralateral rotations and the number of ipsilateral rotations. The net number of rotations was 13.6±8.7 turns/h in the sham- operation group, the sham-operation and non-acupoint-acupunctured group was 8.0±6.3 turns/h, and the sham-operation and Zusanli-acupuntured group was 12.8±9.5. Under normal conditions, acupuncture exerted no significant effect on the net number of rotations.

However, the net number of rotations was increased significantly to 202.8±23.6 turns/h after 6-OHDA injection into the striatum. Moreover, the increased net number of rotations induced by 6-OHDA injection was decreased to 131.7±25.2 turns/h by acupuncture at the Zusanli. However acupuncture at the non-acupoint exerted no significant effect on rotational asymmetry in 6-OHDA-treated rats (192.8±24.1 turns/h).

2. Effect of acupuncture on the survival rate of dopaminergic neurons in the substantia nigra

Photomicrographs of TH-positive cells in the substantia nigra are presented in Fig. 2. The survival rate of TH-positive cells is expressed as percentage of the number of TH-positive cells in the lesion side to the number of TH-positive cells in the intact side. The survival rate was 101.7±4.6% in the sham-operation group, the sham-operation and non-acupoint-acupunctured group was 97.4±5.3%, and the sham-operation and Zusanli-acupunctured group was 103.2± 6.5%. Under normal conditions, acupuncture exerted no significant effect on the survival rate of TH-positive neurons in the substantia nigra.

This survival rate was significantly decreased to 59.5 ±4.6% by 6-OHDA injecting into the striatum. The

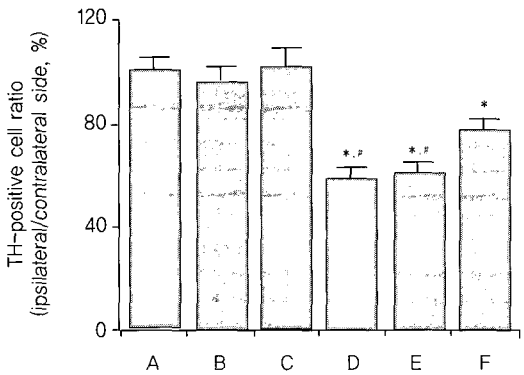

Fig. 2-2. Effect of acupuncture on the survival rate of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra in 6-OHDA-treated rats. Survival rate of TH-positive neurons in the substantia nigra in each group. Values are represented as the mean±S.E.M.. * represents $p < 0.05$ compared to the sham-operation groups. # represents $p < 0.05$ compared to the 6-OHDA-treated and Zusanli-acupuntured group. A) Sham-operation group, (B) sham-operation and non-acupoint-acupunctured group, (C) sham-operation and Zusanli-acupunctured group, (D) 6-OHDA-treated group, (E) 6-OHDA-treated and non-acupoint-acupunctured group, (F) 6-OHDA-treated and Zusanli-acupunctured group.

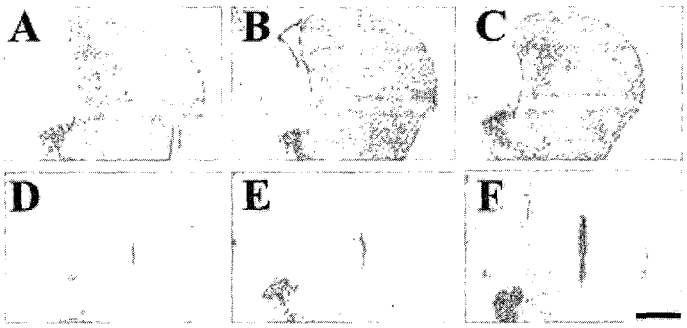

Fig. 3-1. Effect of acupuncture on the value of tyrosine hydroxylase (TH)-positive fiber density in the striatum in 6-OHDA-treated rats. Photomicrographs of TH-positive fibers in the striatum. A scale bar represents 800 μm. (A) Sham-operation group, (B) sham-operation and non-acupoint-acupunctured group, (C) sham-operation and Zusanli-acupunctured group, (D) 6-OHDA-treated group, (E) 6-OHDA-treated and non-acupoint-acupunctured group, (F) 6-OHDA-treated and Zusanli-acupunctured group.

decrease of survival rate induced by 6-OHDA injection was alleviated to 79.2±4.3% by acupuncture at the Zusanli. However, acupuncture at the non-acupoint exerted no significant effect on the survival rate in the substantia nigra of 6-OHDA-treated rats (62.2±3.9%.)

Fig. 2-1 Effect of acupuncture on the survival rate of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra in 6-OHDA-treated rats. Photomicrographs showing TH-specific immunohistochemical staining in the substantia nigra.

The scale bar represents 200 μm. (A) Sham-operation group, (B) sham-operation and non-acupoint-acupunctured group, (C) sham-operation and Zusanli-acupunctured group, (D) 6-OHDA-treated group, (E) 6-OHDA-treated and non-acupoint-acupunctured group, (F) 6-OHDA-treated and Zusanli-acupunctured group.

3. Effect of acupuncture on the value of dopaminergic fiber density in the striatum

Photomicrographs of TH-positive fiber in the striatum are presented in Fig. 3-1. The TH-positive fiber density is expressed as optical density ratio of the lesion side versus the intact side.

The TH-positive fiber density was 1.004±0.011 in the sham-operation group, the sham-operation and non-acupoint- acupunctured group was 0.996±0.024, and the sham-operation and Zusanli-acupunctured group was 0.993± 0.017. Under normal conditions, acupuncture exerted no significant effect on the value of TH-positive fiber density in the striatum.

The TH-positive fiber density was decreased to 0.395 ±0.012 by 6-OHDA-injecting into the striatum. This decreased TH-positive fiber density induced by 6-OHDA was increased to 0.640±0.022 by acupuncture at the Zusanli. However, acupuncture at the non-acupoint exerted no significant effect on the TH-positive fiber density in the striatum of 6-OHDA-treated rats (0.356±0.032).

Fig. 3-1 Effect of acupuncture on the value of tyrosine hydroxylase (TH)-positive fiber density in the striatum in 6-OHDA-treated rats. Photomicrographs of TH-positive fibers in the striatum.

A scale bar represents 800 μm. (A) Sham-operation group, (B) sham-operation and non-acupoint-acupunctured group, (C) sham-operation and Zusanli-acupunctured group, (D) 6-OHDA-treated group, (E) 6-OHDA-treated and non-acupoint-acupunctured group, (F) 6-OHDA-treated and Zusanli-acupunctured group.

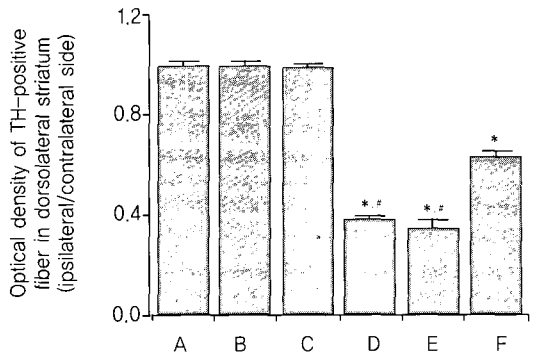

Fig. 3-2. Effect of acupuncture on the value of tyrosine hydroxylase (TH)-positive fiber density in the striatum in 6-OHDA-treated rats. The mean values of TH-positive fibers in each group. Values are presented as the mean ㎛ S.E.M.. * represents $p<0.05$ compared to the sham-operation groups. # represents $p<0.05$ compared to the 6-OHDA-treated and Zusanli-acupuntured group. (A) Sham-operation group, (B) sham-operation and non-acupoint-acupunctured group, (C) sham-operation and Zusanli-acupunctured group, (D) 6-OHDA-treated group, (E) 6-OHDA-treated and non-acupoint-acupunctured group, (F) 6-OHDA-treated and Zusanli-acupunctured group.

Discussion

In the present study, acupuncture treatment exerted no significant effect on the survival of nigrostriatal dopaminergic neurons in non-6-OHDA-treated rats.

In clinical practice, acupuncture treatment at specific acupoints is known to be effective for symptom improvement in Parkinson's disease. In a recent animal study, it was found that electroacupuncture at Dazhui (GV14) and Baihui (GV20) enhances the survival of dopaminergic neurons in the substantia nigra of rats with medial forebrain bundle transection[19-20]. Park et al.18) showed that acupuncture at the Yanglingquan (GB34) and Taichong (LR3) reduces the degeneration of dopaminergic neurons induced by 6-OHDA in rats. Our study also shows that acupuncture stimulation at Zusanli exerted a protective effect against dopaminergic neuronal cell body loss in the substantia nigra and against dopaminergic neuronal fiber loss in the striatum of 6-OHDA-treated rats.

6-OHDA is a neurotoxin that causes the degeneration of dopaminergic neurons, and it has been used to induce rodent model of Parkinson's disease. The neurotoxicity of 6-OHDA is associated with the production of free radicals[27-28], which initiate a death-related cascade in neurons[26]. 6-OHDA is also known to induce apoptotic neuronal cell in the substantia nigra and to reduce the number of dopaminergic neurons[2].

In the present study, the intrastriatal injection of 6-OHDA induced extensive losses in the number of dopaminergic neurons in the substantia nigra and dopaminergic fibers in the striatum, and induced characteristic motor dysfunction as evidence by rotational asymmetry.

Dopaminergic neuronal loss was significantly reduced by acupuncture at the ST36 acupoint in rats with Parkinson's disease. However, acupuncture at a non-acupoint caused no significant effect in the striatum of 6-OHDA-treated rats. This cell rescue by acupuncture at Zusanli was accompanied by a significant recovery in apomorphine-induced motor behavior. Performance in motor tests depends on the extents of striatal dopaminergic innervation and dopaminergic tone in the partially lesioned area[29-31]. The present results show that acupuncture treatment at Zusanli exerts a protective effect on 6-OHDA-induced

injury to nigrostriatal dopaminergic neurons.

The Zusanli is a well documented acupoint in animals[22-23] and human[32], and is often used for modulating gastrointestinal functions and relieving pain[33]. It was reported that acupuncture at Zusanli reduces lipid peroxidation level after stroke[34], and inhibits brain damage in stroke patients[35]. Recent studies have shown that the stimulation of this acupoint enhances neuronal activity in various brain regions32, [36-38], and activates catecholaminergic neurons in the rat brainstem[39].

In this study, we found that acupuncture treatment at Zusanli suppressed nigrostriatal dopaminergic neuronal cell death in a rodent model of Parkinson's disease. Moreover, it suggests that acupuncture, especially at Zusanli, provides a useful strategy for the treatment of Parkinson's disease.

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