EFFECT OF ACUPUNCTURE ON NITRIC OXIDE SYNTHASE EXPRESSION IN CEREBRAL CORTEX OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT:
Effect of acupuncture on the expressions of nitric oxide synthase (NOS) and neuronal NOS (nNOS) in the cerebral cortex of streptozotocin (STZ)-induced diabetic rats was investigated. Animals were divided into four groups; the control group, the nondiabetic and acupunctured group, the STZ-induced-diabetes group, and the STZ-induced diabetic and acupunctured group. To produce the diabetic animal model, a single intraperitoneal injection of STZ (50 mg/kg) was given to each animal; animals of the nondiabetic groups received equivalent amounts of normal saline, also via intraperitoneal injection. From the results, acupuncture was shown to increase the numbers of nicotinamide adenine dinucleotide phosphate-diaphorase-positive and nNOS-positive neurons in STZ-induced diabetic rats. From the present study, it may be suggested that acupuncture modulates NOS and nNOS expressions in the cerebral cortex under diabetic conditions.

KEY WORDS: Acupuncture; Streptozotocin; Diabetes; Nicotinamide adenine dinucleotide phosphate-diaphorase; Neuronal nitric oxide synthase; Cerebral cortex
INTRODUCTION

Nitric oxide (NO), endogenously generated from L-arginine by nitric oxide synthase (NOS), is a free radical with signaling functions in the central nervous system (CNS). It has been known to play an important role in the regulation of basal cerebral circulation and has been implicated in numerous physiological and pathological processes in the brain [1]. Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is a histochemical marker specific for NOS in the CNS. Neurons containing NADPH-d have been reported to be relatively resistant to various toxic insults and neurodegenerative disorders [2]. Several isoforms of NOS exist that fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) [3]. Of these, nNOS is mainly expressed in the CNS and has been implicated in signal transmission and synaptic plasticity in neuronal cells [4].

Diabetes mellitus is one of the most common serious metabolic disorders in humans. In addition to the diabetic condition itself, numerous secondary complications are associated with the illness [5]. In diabetic rats, suppressed NOS activity was observed in the Langerhans islet cells and in the total pancreatic homogenate as compared to the normal rats [6]. Recently, decreased NOS expression in the cerebral cortex [7] and cerebellum [8] of streptozotocin (STZ)-induced diabetic rats has also been reported.

Acupuncture has been used as a clinical treatment for various diseases in Oriental medicine. Acupuncture treatment is known to possess many effects, such as analgesia, promotion of homeostasis, and changes in the microcirculatory network as well as improvement in brain circulation [9-11]. Acupuncture has also been used to relieve symptoms of diabetes mellitus [9,12].

In the present study, acupuncture-induced changes in the expressions of NOS and nNOS in the cerebral cortex of STZ-induced diabetic rats were investigated via NADPH-d histochemistry and nNOS immunohistochemistry.

MATERIALS AND METHODS

Animal Preparation

Male Sprague-Dawley (S-D) rats weighing 200 ± 10 g (6 weeks in age) were used for the experiment. Each animal was housed at a controlled temperature (20 ± 2°C) and was maintained under light-dark cycles, each cycle consisting of 12 h of light and 12 h of darkness (light on from 07:00 h to 19:00 h). Food and water were made available ad libitum. The experimental procedures were performed in accordance with the animal care guidelines of NIH and the Korean Academy of Medical Sciences. Animals were divided into four groups: the control group, the nondiabetic and acupunctured group, the STZ-induced diabetes group, and the STZ-induced diabetic and acupunctured group (n = 5 in each group). To produce the diabetic animal model, a single intraperitoneal injection of STZ (50 mg/kg; Sigma, St. Louis, MO, USA)
was given to each animal; animals of the nondiabetic groups received equivalent amounts of normal saline, also via intraperitoneal injection. Blood glucose levels were determined 2 days after injection of STZ using a blood glucose tester (Arkay, Kyoto, Japan). Only these animals that exhibited blood glucose levels of 300 mg/dl or higher were used in this study.

**Acupuncture Methods**

For acupuncture stimulation, stainless acupuncture needles of 0.3 mm diameter were bilaterally inserted into the locus of ST 36 (Zusanli), located 5 mm lateral and distal to the anterior tubercle of the tibia, and were left in place for 20 min [10]. In the acupunctured groups, acupunctural treatment was given to each animal twice daily (10:00 a.m. and 6:00 p.m.) for 5 consecutive days starting on the second day after STZ administration. All animals were sacrificed on the 8th day after commencement of the experiment.

![Image](image_url)

Fig. 1. The figure represents the ST 36 (Zusanli) acupoint (Arrow).

**Tissue Preparation**

For the sacrificial process, animals were first fully anesthetized with Zoletil® (10 mg/kg, i.p.; Vibac, Carros, France), then transcardially perfused with 50 mM phosphate-buffered saline (PBS), and then fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). The brains were then removed, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40 μm thickness were made with a freezing microtome (Leica, Nussloch, Germany).

**NADPH-d Histochemistry**

For visualization of NADPH-d activity, sections were stained according to a previously described protocol [13]. In brief, free-floating sections were incubated at 37°C for 1 h in 100 mM PB containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium, and 0.1 mg/ml β-NADPH (Sigma, St. Louis, MO, USA). The sections were then washed three times with PBS and were mounted onto gelatine-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount®.
Fig. 2. The figure represents dissecting procedure of brain (left panel) and dissected brain (right panel). Dotted line represents approximate location of the cross section of Fig. 3.

Fig. 3. The figures show cross sectional areas of the brain cortex. A, Bregma -3.60 mm; B, Bregma -4.80 mm.

**nNOS Immunohistochemistry**

For nNOS immunohistochemistry, free-floating sections were incubated for 48 h in PBS (4°C) containing anti-nNOS antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 0.3% Triton X-100, 0.05% bovine serum albumin and 1.5% normal horse serum. The sections were then incubated with biotinylated anti-mouse IgG secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h and then with an avidin-biotin-peroxidase complex (1:100, Vector Laboratories, Burlingame, CA, USA) for another 1 h at room temperature. The sections were stained by incubating with 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 for 3 min. Analysis of the stained sections was made using the atlas by Paxinos and Watson [14]. Results were obtained as mean number of NADPH-d-positive neurons or nNOS-positive neurons per section.
Statistical Analysis

Statistical differences were determined by one-way ANOVA followed by Scheffe's post-hoc analysis, and results were expressed as mean ± S.E.M. Differences were considered significant for $P < 0.05$.

RESULTS

In the experimental animals, NADPH-d-positive and nNOS-positive neurons were observed in the cerebral cortex (Fig. 3). The number of NADPH-d-positive neurons in the cerebral cortex is shown in Table 1. The number of NADPH-d-positive neurons in cerebral cortex was decreased in STZ-induced diabetic rats. Acupuncture treatment at Zusanli acupoint increased NADPH-d expression in cerebral cortex significantly under the diabetic conditions, while acupuncture conditions exerted no specific effect on NADPH-d expression under the normal conditions.

The number of nNOS-positive neurons in the cerebral cortex is shown in Table 2. The number of nNOS-positive neurons in cerebral cortex was decreased in STZ-induced diabetic rats. Acupuncture treatment at Zusanli acupoint increased nNOS expression in cerebral cortex significantly under the diabetic conditions, while acupuncture conditions exerted no specific effect on nNOS expression under the normal conditions.

Fig. 4. Photomicrography of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)-positive cell (left panel) and neuronal nitric oxide synthase (nNOS)-positive cell (right panel). Scale bar represents 25 μm.
Table 1. Number of NADPH-d-positive neurons in several regions of the cerebral cortex in each group.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Nondiabetic and acupuncture group</th>
<th>STZ-induced-diabetes group</th>
<th>STZ-induced diabetic and acupuncture group</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSG/RSA</td>
<td>2.51 ± 0.26</td>
<td>2.82 ± 0.43</td>
<td>1.46 ± 0.17*</td>
<td>2.23 ± 0.22*</td>
</tr>
<tr>
<td>Fr 1/2</td>
<td>2.31 ± 0.22</td>
<td>2.59 ± 1.33</td>
<td>2.18 ± 0.18</td>
<td>2.27 ± 0.27</td>
</tr>
<tr>
<td>HL</td>
<td>16.00 ± 0.79</td>
<td>12.82 ± 0.91*</td>
<td>8.82 ± 0.67*</td>
<td>15.91 ± 0.77*</td>
</tr>
<tr>
<td>Par 1</td>
<td>13.26 ± 0.98</td>
<td>9.06 ± 1.18*</td>
<td>9.96 ± 0.88*</td>
<td>14.86 ± 0.78*</td>
</tr>
<tr>
<td>Par2</td>
<td>13.08 ± 0.59</td>
<td>10.92 ± 0.68*</td>
<td>7.77 ± 0.66*</td>
<td>11.23 ± 0.63*</td>
</tr>
<tr>
<td>PRh</td>
<td>12.63 ± 0.91</td>
<td>10.56 ± 0.90</td>
<td>11.00 ± 0.67</td>
<td>14.32 ± 0.59*</td>
</tr>
<tr>
<td>Oc2MM</td>
<td>6.06 ± 0.39</td>
<td>6.69 ± 0.42</td>
<td>3.91 ± 0.25*</td>
<td>14.32 ± 0.59*</td>
</tr>
<tr>
<td>Oc2ML</td>
<td>6.06 ± 0.38</td>
<td>4.88 ± 0.39</td>
<td>5.84 ± 0.38</td>
<td>6.00 ± 0.41</td>
</tr>
<tr>
<td>Oc2L</td>
<td>6.50 ± 1.86</td>
<td>6.44 ± 0.40</td>
<td>3.90 ± 0.31*</td>
<td>4.24 ± 0.38*</td>
</tr>
<tr>
<td>Te 1</td>
<td>16.61 ± 0.70</td>
<td>16.13 ± 0.70</td>
<td>8.26 ± 0.34*</td>
<td>16.13 ± 0.70*</td>
</tr>
<tr>
<td>Te 3</td>
<td>6.61 ± 0.56</td>
<td>7.19 ± 0.58</td>
<td>4.40 ± 0.34*</td>
<td>5.68 ± 0.34*</td>
</tr>
</tbody>
</table>

RSA, retrosplenial agranular cortex; RSG, retrosplenial granular cortex; Fr1/2, frontal cortex area 1 and 2; HL, hindlimb area of cortex; Par 1, parietal cortex areas 1; Par 2, Parietal cortex area 2; PRh, perirhinal cortex; Oc2MM, occipital cortex area 2 mediolateral; Oc2ML, occipital cortex area 2 lateral; Te 1, temporal cortex area 1; Te 3, temporal cortex area 3. * means $P < 0.05$ compared to the control group. # means $P < 0.05$ compared to the streptozotocin (STZ)-induced-diabetes group.
Table 2. Number of nNOS-positive neurons in several regions of the cerebral cortex in each group.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Nondiabetic and acupunctured group</th>
<th>STZ-induced diabetes group</th>
<th>STZ-induced diabetic and acupuncture group</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSG/RSA</td>
<td>1.96 ± 0.22</td>
<td>2.39 ± 0.23</td>
<td>1.03 ± 0.13*</td>
<td>2.15 ± 0.19*</td>
</tr>
<tr>
<td>Fr 1/2</td>
<td>3.96 ± 0.31</td>
<td>3.46 ± 0.31</td>
<td>1.66 ± 0.18*</td>
<td>3.75 ± 0.23*</td>
</tr>
<tr>
<td>HL</td>
<td>17.89 ± 0.72</td>
<td>16.46 ± 0.39</td>
<td>12.28 ± 0.74*</td>
<td>16.50 ± 0.61*</td>
</tr>
<tr>
<td>Par 1</td>
<td>16.86 ± 0.58</td>
<td>17.36 ± 0.66</td>
<td>8.72 ± 0.51*</td>
<td>16.50 ± 0.62*</td>
</tr>
<tr>
<td>Par2</td>
<td>17.79 ± 1.06</td>
<td>16.54 ± 0.64</td>
<td>8.72 ± 0.44*</td>
<td>14.08 ± 0.52*</td>
</tr>
<tr>
<td>PRh</td>
<td>15.57 ± 0.61</td>
<td>14.54 ± 0.50</td>
<td>9.79 ± 0.51*</td>
<td>16.46 ± 0.50*</td>
</tr>
<tr>
<td>Oc2MM</td>
<td>6.47 ± 0.35</td>
<td>5.91 ± 0.31</td>
<td>4.05 ± 0.32*</td>
<td>6.24 ± 0.42*</td>
</tr>
<tr>
<td>Oc2ML</td>
<td>6.32 ± 0.33</td>
<td>5.91 ± 0.31</td>
<td>4.13 ± 0.43*</td>
<td>6.67 ± 0.35*</td>
</tr>
<tr>
<td>Oc2L</td>
<td>6.39 ± 0.28</td>
<td>5.36 ± 0.30*</td>
<td>5.19 ± 0.4*</td>
<td>6.71 ± 0.24*</td>
</tr>
<tr>
<td>Te 1</td>
<td>18.11 ± 0.66</td>
<td>17.96 ± 0.46</td>
<td>13.69 ± 0.62</td>
<td>16.29 ± 0.54*</td>
</tr>
<tr>
<td>Te 3</td>
<td>6.11 ± 0.27</td>
<td>6.09 ± 0.32</td>
<td>5.31 ± 0.37*</td>
<td>7.048 ± 0.46*</td>
</tr>
</tbody>
</table>

RSA, retrosplenial agranular cortex; RSG, retrosplenial granular cortex; Fr1/2, frontal cortex area 1 and 2; HL, hindlimb area of cortex; Par 1, parietal cortex areas 1; Par 2, Parietal cortex area 2; PRh, perihinal cortex; Oc2MM, occipital cortex area 2 mediolateral; Oc2ML, occipital cortex area 2 mediolateral; Oc2L, occipital cortex area 2 lateral; Te 1, temporal cortex area 1; Te 3, temporal cortex area 3. * means $P < 0.05$ compared to the control group. # means $P < 0.05$ compared to the streptozotocin (STZ)-induced-diabetes group.
DISCUSSION

Diabetes mellitus is a disorder of glucose metabolism caused by a partial or complete deficiency of insulin, and it is associated with chronic complications such as neuropathy and vasculopathy [15]. Of particular interest is deficits in cerebral metabolism observed in diabetic rats, which seem to be the end product of a multifactorial process involving chronic cerebral hyperglycemia [5,16]. In the present study, the effect of acupuncture stimulation at the Zusanli acupoint on the expressions of NOS and nNOS in the cerebral cortex was investigated. Zusanli is a well known acupoint in animals [10,17,18] and humans [19]. The Zusanli acupoint has been widely used in Oriental medicine for treatment of diabetes [20].

In the stained sections, NADPH-d-positive neurons were observed as black entities with features corresponding to perikarya, dendrites and axon, while nNOS neurons were stained brown. From the results of the present study, the numbers of neurons containing NOS or nNOS in the STZ-induced diabetes group were significantly decreased in several regions of the cerebral cortex compared to those of the control group. Similar results were observed in the platelets of insulin-dependent diabetes mellitus patients [21], and in the cerebrocortex of STZ-induced diabetic rats [7]. Decrease in NOS activity is thought to increase vessel resistance, which results in decrease of blood flow [22]. Chang et al. [9] reported that electroacupuncture stimulation at the Zhongwan acupoint could lower plasma glucose concentrations regardless of the presence of hypoglycemia through an enhancement in β-endorphin secretion. In addition, it has recently been reported that repeated electroacupuncture alters NADPH-d and nNOS activity in the brainstem of rats with spontaneous hypertension [18].

In the present study, acupuncture treatment was shown to exert no significant effect on the expression of NOS and nNOS in the cerebral cortex under the normal conditions. On the other hand, NOS and nNOS expressions were significantly increased by acupuncture treatment under the diabetic conditions.

In conclusion, it may be suggested that acupuncture modulates NOS and nNOS activity in the cerebral cortex under the diabetic conditions; further study, however, is needed to reveal the complete mechanism responsible for the observed effects.

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