ACUPUNCTURE DECREASES NEUROPEPTIDE Y EXPRESSION IN THE HYPOTHALAMUS OF RATS WITH STREPTOZOTOCIN-INDUCED DIABETES

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ABSTRACT:
It has been known that acupuncture has various effects such as analgesia, promotion of homeostasis, improvements in brain circulation, and rectification of internal disorders. Neuropeptide Y (NPY), a 36-amino-acid peptide, is known to increase appetite. In the present study, the effect of acupuncture stimulation at Zusanli (St.36) on NPY expression in the Streptozotocin (STZ)-induced diabetic rats was investigated via immunohistochemistry. Increased NPY expression was detected in both the Arcuate nucleus (ARN) and the Paraventricular nucleus (PVN) of the Hypothalamus in rats with in STZ-induced diabetes. Needling on Zusanli resulted in decreased NPY levels in both the ARN and PVN of diabetic rats. The present study shows that acupuncture suppressed NPY expression in the ARN and PVN of the Hypothalamus in STZ-induced diabetic rats, suggesting the possibility that acupuncture treatment is effective in curbing the hyperphagia of diabetes.

Key Words: Acupuncture; Diabetes; Immunohistochemistry; Hypothalamus; Neuropeptide Y; Hyperphagia; Streptozotocin
INTRODUCTION

Diabetes mellitus is one of the most common metabolic disorders in humans. In addition to the diabetic condition itself, numerous secondary complications are associated with the illness [1]. It is characterized by marked hyperphagia, reduced thermogenesis, and impaired secretion of most pituitary hormones [2]. As the Hypothalamus appears to be important in regulating food intake and energy balance, these energetic and neuroendocrine disturbances seen in diabetes may be mediated by changes in the levels and distributions of specific hypothalamic neuron and neurotransmitters [3]. Neuropeptide Y (NPY), a 36-amino-acid peptide related to pancreatic polypeptide, is concentrated in appetite-regulating areas of the Hypothalamus. NPY was first isolated from porcine brain in 1982. NPY is synthesized in neuron of the Arcuate nucleus which project mainly to the Paraventricular nucleus and the dorsomedial nuclei [4]. Neuropeptide Y injected into the Paraventricular nuclei or other hypothalamic sites induces intense carbohydrate-prefering hyperphagia, with a fall in sympathetically-mediated brown adipose tissue activity, and its long term administration leads to obesity [5]; indeed, it is one of the most potent appetite stimulants known [3].

It has been known that acupuncture treatment possesses various effects such as analgesia, promotion of homeostasis, improvements in brain circulation, and rectification of internal disorders [6-11]. Acupuncture has also been used to relieve symptoms of diabetes mellitus for many years in Oriental medicine [12,13]. Our previous study showed that auricular acupuncture decreases NPY expression in the Hypothalamus of food-deprived rats [8]. It has been known that acupuncture stimulation on the acupoints is more effective than that of non-acupoints. Wang et al. suggested that the Hanyan (G4), Xuanlu (G5), Xuanli (G6), and Qubin (G7) acupoints are effective for the functional recovery and that enhanced expression of vascular endothelial growth factor (VEGF) may play a certain role in recovery process after stroke [14]. Of particular interest is Zusanli acupoint (SI.36), which has widely been applied for the treatment of the various diseases including pain and stroke [9,15-17]. Zusanli acupoint (ST.36) has well been documented in animals and humans [6,9,17-20]. Omura suggested that the meridian-like network seems to be specialized channel which can propagate some type of information in electro-magnetic field to regulate some of the body functions throughout the body [21]. Omura et al. also showed that acupuncture on the Zusanli acupoint (ST.36) on normal subjects increased the telomere levels up to a maximum of more than two times their telomere levels prior to the treatment. (22).

In the present study, the modulatory effect of acupuncture on hypothalamic NPY expression in rats with Streptozotocin (STZ)-induced diabetes was investigated via immunohistochemistry.

MATERIALS AND METHODS

Animal Preparation

Male Sprague-Dawley rats weighing 200 ± 10 g were used for the experiment. Each animal was housed at a controlled temperature (20 ± 2°C) and was maintained in light-dark cycles, each cycle consisting of 12 h of light and 12 h of darkness (lights on from 07:00 h to 19:00 h) with food and water made available ad libitum for 7 days prior to the commencement of the experiment. 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 acupunctured group, the STZ-induced-diabetes group, and the STZ-induced-diabetes-and-acupuncture group (n = 5 in each group). To produce an animal model for diabetes, a single intraperitoneal injection of STZ (50 mg/kg) was given to each animal. The control animals received normal saline injections. Blood glucose levels were determined 2 days after STZ injection using a blood glucose tester (Arkray, Kyoto, Japan). Only the animals that exhibited blood glucose level of 300 mg/dL or above were used in the experimental groups. Two days after STZ administration, both of the acupunctured groups were given acupuncture treatment twice daily (10:00 a.m. and 5:00 p.m.) for 5 consecutive days.
Acupuncture Methods
For acupuncture stimulation, stainless acupuncture needles of 0.3 mm diameter were inserted bilaterally into the locus of Zusanli (ST.36), located 5 mm lateral and distal to the anterior tubercle of the tibia, and were left in place for 20 min [9]. All of the animals were sacrificed on the 8th day after the commencement of the experiment.

Tissue Preparation
At the beginning of the sacrificial procedure, animals were weighed and overdosed with sodium pentobarbital. After a complete lack of response was observed, the rats were transcardially perfused with 0.05 M phosphate-buffered saline (PBS) and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The brains were dissected, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Serial coronal sections of 40 μm thickness were made using a freezing microtome (Leica, Nussloch, Germany).

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

Immunohistochemistry
Eight sections on average were collected from each brain for immunohistochemistry. For the immunohistochemical procedure, free-floating tissue sections were washed twice for 15 min in 0.05 M PBS, and then permeabilized in 0.2% Triton X-100 for 30 min. After washing twice with PBS, the sections were incubated overnight with rabbit anti-NPY antiserum (DiaSorin, MN) at a dilution ratio of 1:4000. The sections were washed twice in PBS and incubated for 1 h with a biotinylated anti-rabbit antibody. Bound secondary antibody was then amplified with the Vector Elite ABC kit (Vector Laboratories, Burlingame, CA). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.05% diaminobenzidine.

Data Analysis
The intensities of NPY-specific staining were assessed in a quantitative fashion according to a microdensitometrical method based on optical density (mean gray scale) using an image analyzer (Multiscan, Fullerton, CA). Before starting the image analysis, the light source
was adjusted to the brightness generating the best possible contrast between positive- and negative-staining cells.

**Statistical Analysis**

Results were expressed as mean ± S.E.M. and were analyzed by Student's t-test using SPSS (version 7.5). Differences were considered statistically significant at $P < 0.05$.

**RESULTS**

Fig. 2 shows cross sectional area of the rat Hypothalamus. The results are shown in Table 1. Fig. 3 represents molecular structure of the Neuropeptide Y. The NPY level in the ARN and PVN was assessed by optical density measurement.

In the control animals, typical levels of NPY-immunoreactivity were observed in the ARN and PVN. Compared to the control rats, higher levels of NPY were detected in both the ARN and PVN of the diabetic rats. This observation is consistent with those from previous reports, that NPY-positive neurons of the arcuato-paraventricular pathway are stimulated under conditions of negative energy balance, most notably insulin-deficient diabetes and starvation, and that NPY levels, NPY mRNA levels, and NPY secretion within the PVN are increased under such conditions [2,8,10,14-18]. Increased activity of the NPY neurons in the ARN may therefore be responsible for the hyperphagia of diabetes and some of the major neuroendocrine alterations associated with this disorder.

![Diagram](image.png)

**Fig. 2.** The figure shows cross sectional areas of the rat Hypothalamus under -2.12 mm from the Bregma. A scale bar represents 1 mm.
Fig. 3. Molecular structure of the Neuropeptide Y.

Table 1. Staining intensities of NPY-positive neurons in the ARN and PVN.

<table>
<thead>
<tr>
<th>Group</th>
<th>ARN</th>
<th>PVN</th>
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<tbody>
<tr>
<td>Control</td>
<td>70.39 ± 3.17</td>
<td>71.90 ± 2.83</td>
</tr>
<tr>
<td>Nondiabetic acupunctured</td>
<td>89.04 ± 3.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.93 ± 2.27&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Streptozotocin-induced-diabetes</td>
<td>92.51 ± 1.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.73 ± 3.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Streptozotocin-induced-diabetes-and-acupunctured</td>
<td>73.04 ± 3.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.91 ± 2.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data represents mean ± S.E.M. (n = 5 in each group). <sup>a</sup>means P < 0.05 compared to the control group; <sup>b</sup> means P < 0.05 compared to the Streptozotocin-induced-diabetes group (Student’s t-test). ARN, Arcuate nucleus; PVN, Paraventricular nucleus.
Fig. 4. Distribution of NPY-immunoreactive neurons in the Arcuate nucleus (ARN). Control group (A), the nondiabetic and acupunctured group (B), the Streptozotocin-induced diabetes group (C), and Streptozotocin-induced-diabetes-and-acupunctured group (D). A scale bar represents 250 µm.

Fig. 5. Distribution of NPY-immunoreactive neurons in the Paraventricular nucleus (PVN). Control group (A), the nondiabetic and acupunctured group (B), the Streptozotocin-induced diabetes group (C), and Streptozotocin-induced-diabetes-and-acupunctured group (D). A scale bar represents 250 µm.
DISCUSSION

NPY is one of the most important neurotransmitters in the hypothalamic neural circuits that regulate food intake and body weight. NPY stimulates feeding desire and decreases energy expenditure [4]. Administration of NPY resulted in robust and sustained increase in food intake in experimental animals [5]. NPY is a potent orexigenic agent and its release is increased during starvation and under diabetic conditions [2,3]. Increases in both biosynthesis and release of NPY along the discrete neural pathways constitute a specific hypothalamic response to starvation [8,25]. The various sub-regions of the Hypothalamus play important roles in the regulation of food intake and energy expenditure.

Peripheral administration of STZ causes marked hyperglycemia, hyperphagia, and polydipsia, and insulin treatment reverses these symptoms [28]. Numerous studies have shown that insulin deficiency-induced diabetes causes alteration in the activities of hypothalamic transmitters and neuropeptides [28,29]. In order to investigate the effect of acupuncture treatment on diabetes, animals of the acupunctured groups, both nondiabetic and diabetic, were stimulated on Zusanli (ST.36) twice daily during the experiment. NPY levels were increased in both the ARN and PVN in the nondiabetic rats by acupuncture stimulation (Table I). This observation is consistent with our previous report that acupuncture stimulation acts as stress on rats under normal conditions [8]. It has been reported that stress increases NPY mRNA levels in the ARN [30]. In contrast, NPY levels in both the ARN and PVN of the STZ-induced-diabetes rats were significantly decreased by acupuncture treatment (Table I). This suggests that even though acupuncture might act as stress under normal conditions, such stimulation is effective in attenuating the desire for food under diabetic condition.

Zhang et al. reported that stimulation of acupoints, Zusanli/Sanyinjiao (ST36/SP6) or Yanglingquan/Chengshan (GB34/BL57), in the same spinal segments induced distinct through overlapped cerebral response patterns. These results suggest the existence of acupoint specificity [20]. Recent studies reported that manual acupuncture on the Zusanli acupoint (ST.36) shows analgesic effect [17] and activates limbic area [19]. Lee et al. reported that the analgesic effect of acupuncture on the Zusanli acupoint (ST.36) is closely related with the cholecystokinin-A receptor expression [31]. Jang et al. reported that acupuncture on the Zusanli acupoint (ST.36) alleviated ischemia-induced apoptosis, suggesting that acupuncture treatment may aid in the recovery following ischemic cerebral injury [32]. Chen and Ma demonstrate that a blockade of neuronal conduction in the gracile nucleus inhibited the cardiovascular responses to electroacupuncture on the Zusanli acupoint. The hypotensive and bradycardiac response induced by electroacupuncture on the Zusanli acupoint (ST.36) was modified by influences of L-arginine-derived NO synthesis in the gracile nucleus [33].

In many studies, the effectiveness and action of the acupoints in relation to specific diseases have been suggested. However, the exact mechanism of acupuncture treatment and the role of acupoints have not been clarified yet.

The present results demonstrate that acupuncture treatment at Zusanli (ST.36) acupoint suppressed NPY expression in the Hypothalamus of STZ-induced-diabetes rats. This finding suggests the possibility that acupuncture treatment is effective in curbing the hyperphagia of diabetes; however, further study is needed to reveal the complete mechanism behind this effect.

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