

# 침자가 LPS를 주입한 흰쥐 시상하부 방실핵의 신경활성에 미치는 영향

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## Effects of Acupuncture on the Neuronal Activation of Paraventricular Nucleus of Hypothalamus in Lipopolysaccharide-injected Rats

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### 국문초록

본 연구에서는 침의 면역조절작용을 통한 염증반응 억제효과를 연구하기 위하여 내독소를 주입한 흰쥐의 시상하부에서 염증반응의 증추인 방실핵의 신경활성에 미치는 영향을 관찰하였다. 흰쥐의 미정맥에 LPS와 생리식염수를 각각 주입하고 군에 따라 양측 소부(HT8)나 족삼리(ST36)에 1분간 침치료를 각각 시행하였다. C-Fos는 신경활성을 자극하는 초기단계에 발현되는 유전자로서 신경계의 특정부위의 활성도를 측정하는 지표로 널리 사용되고 있다. 본 연구자는 침자극이 LPS로 인한 염증반응에 미치는 영향과 그 기전을 알아보기 위해 면역조직화학염색의 방법을 이용하여 대뇌 시상하부의 방실핵에서 c-Fos 면역활성을 측정하였다.

LPS를 주입한 군의 방실핵에서 생리식염수를 주입한 군에 비해 c-Fos 면역활성이 유의하게 증가한 반면 소부에 자침했을 때 LPS에 의해 증가된 c-Fos 면역활성이 유의하게 감소하였다. 족삼리에 자침한 군에서는 유의한 변화가 나타나지 않았다.

결론적으로 소부 침치료를 LPS로 인해 증가된 방실핵의 신경활성을 효과적으로 감소시켰고 이는 침의 면역조절 및 탁월한 염증억제 효과를 보여주는 결과일 뿐 아니라 침의 인체 항상성 유지를 통한 치료기전에 대한 향후 연구의 중요한 실마리를 제공해주고 있다고 사료된다.

**Keywords :** Acupuncture, Lipopolysaccharide, Paraventricular nucleus, Neuronal activation, Immunomodulation.

## 1. Introduction

Acupuncture has long been renowned for its effective therapeutic benefits, such as relief of pain, immunomodulation and treatment of internal organic disorders in

Oriental medicine<sup>16,20,30</sup>). According to the theories of Oriental medicine, acupuncture acts on human body to sustain the Yin and Yang balance by regulating Qi, and finally displays its efficacy on various diseases<sup>11</sup>.

Particularly, many researchers have reported studies on the immunomodulatory effect of acupuncture<sup>3,32</sup>). For example, modulatory effect of acupuncture on immune

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function has been reported in the traumatized rats<sup>5,7,8)</sup>. In addition, other researchers observed that several immune-related indexes such as the natural killer cell activity, interferon- $\gamma$  and interleukin-2 increased in normal animals after acupuncture treatment<sup>27,39,40)</sup>.

Lipopolysaccharide (LPS) is an endotoxin derived from gram-negative bacterial cell wall. It has been used to induce the bacterial infection-like symptom in animals by central or peripheral injection. This infectious condition, including fever, is known to be activated by pro-inflammatory cytokines - e.g. interleukin- $1\beta$ , tumor necrosis factor- $\alpha$ - that stimulate the central immune regulatory center, the hypothalamus<sup>10,15)</sup>. Stimulating hypothalamus via cytokine-induced pathway could be followed by the activation of hypothalamic-pituitary- adrenal (HPA) axis, which induces systemic symptoms such as decrease in feeding, drinking, social interactions and alterations in brain neurochemistry<sup>15)</sup>. In fact, these cytokines activate the HPA axis mainly by stimulating the neurons in the paraventricular nucleus (PVN)<sup>10,18)</sup>.

PVN has been known to represent a center for homeostasis. It contains specific neuronal projections to endocrine and autonomic control sites<sup>33)</sup> and involves in the acute phase reaction of inflammatory response<sup>18,36)</sup>. As a main integrative part of hypothalamus, the PVN is known to be activated in response to immune stress, e.g. via the systemic injection of endotoxin, and to act on the anterior pituitary gland by secreting corticotropin releasing factor (CRF) to stimulate the release of adrenocorticotropin hormone (ACTH)<sup>2,17)</sup>. It is well known that

peripheral (i.v. or i.p.) injection of LPS stimulates the neurons in the PVN<sup>10,25)</sup>, and in turn, increases the plasma CRF and ACTH through the activation of HPA axis<sup>12,13,26,34)</sup>.

C-Fos, which is an immediate early gene expressed after different kinds of stimulation, has been used as neuronal marker<sup>14,29)</sup>.

Integration of above known facts suggests a hypothesis that the anti-inflammatory effects of acupuncture could be mediated by the activation of the PVN neurons. Therefore, I have carried out this study to investigate the effects of acupuncture on the neuronal activation of PVN in the LPS-injected rats using c-Fos immunohistochemistry.

## II. Materials and methods

### 1. Animals

Male Sprague-Dawley rats (n = 30) weighing 300-350 g were used in this experiment. All animals were supplied from Korean Experimental Animal Center and treated according to NIH guideline.

### 2. Materials

Lipopolysaccharide (LPS; E. coli, 055:B5, Sigma, USA) was used for animal infection model. For acupuncture treatment, sterilized needles (stainless steel, 0.25 mm o.d., Dongbang acupuncture INC, Korea) were used.

### 3. LPS injection

LPS (400  $\mu\text{g}/\text{kg}$ , i.v.) diluted in normal

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saline was injected to mimic the bacterial infection of rats. Normal saline was injected intravenously for saline control groups.

### 4. Acupuncture treatment

Two acupuncture points were selected for the purpose of treatment and control. The acupuncture point, Sobu (HT8) is located between fourth and fifth metacarpal bones in the palm of forepaw. Control point, Joksamli (ST36) is located 5 mm laterally and distally from the anterior tubercle of the tibia.

HT8 has been used for the treatment of fever-related disorders because of its immunomodulatory effects in Oriental medicine<sup>1,31</sup>. ST36 was selected for control acupoint in this experiment though it was reported to have various other effects<sup>16,19,20</sup>.

Acupuncture needles were bilaterally inserted into each acupoints to a depth of 5 mm and twisted at 2 Hz for 1 min. All treatments were performed 2 hrs after LPS- or saline- injection was treated.

### 5. Immunohistochemistry

Animals were anesthetized by sodium pentobarbital 4 hrs after injection for the preparation of perfusion. After checking for complete lack of response, 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. Brains were dissected, post-fixed in the same fixative overnight, and transferred into 30% sucrose for cryoprotection.

Serial frontal sections up to 40  $\mu$ m thickness were made using a freezing microtome (Shandon, UK). Twelve sections

were collected from each brain for immunohistochemistry, and steeped in storing solution with 30% glycerin and ethylene glycol in 0.2 M PB. For immunohistochemical procedure, free-floating tissue sections were washed three times for 15 min in 0.05 M PBS, and then incubated in 1% H<sub>2</sub>O<sub>2</sub> in 0.05 M PBS for 15 min. After washing it three times with the PBS for the removal of bubbles, sections were incubated in the blocking solution with 1% BSA and 10% goat serum for 1 hr, and subsequently incubated overnight with c-Fos primary antibody (1:20000, Oncogene, MA) at room temperature. After washing it three times with PBS, sections were incubated for 1 hr with biotinylated anti-rabbit antibody (1:100, Vector laboratories, Burlingame, CA). Bound secondary antibody was then amplified with the Vector Elite ABC (Avidin biotin complex) kit for 1 h before washing it three times with PBS. For visualization, the antibody-avidin-biotin-peroxidase complexes were stained using 0.02% 3,3'-diaminobenzidine containing nickel chloride (40 mg/ml) (Nickel-DAB) in 0.05 M Tris buffer (pH 7.6) with 0.03% H<sub>2</sub>O<sub>2</sub> for 3 to 5 min. After sufficient visualization has been achieved, reacted sections were mounted on gelatin-coated slide, and dried overnight.

The intensities of c-Fos-specific staining were assessed in a quantitative fashion according to a microdensitometrical method based on optical density (mean gray scale) using an image analyzer (OPTIMAS version 6.5, Media Cybernetics, MD). Before starting the image analysis, the light source was adjusted to the brightness, which generates the best possible contrast between positive- and negative- staining cells. C-Fos immunoreactivity in the PVN was analyzed

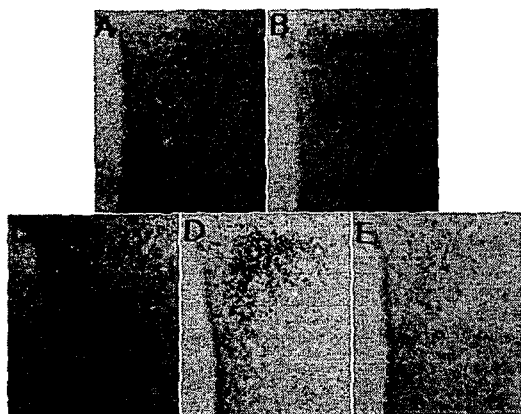


Fig 1. C-Fos immunoreactivity in the same sections of the PVN of hypothalamus 4 hrs after injection in rats.

- A: Group with saline-injection
- B: Group with LPS-injection
- C: Group with acupuncture on HT8 after LPS-injection
- D: Group with acupuncture on ST36 after LPS-injection
- E: Group with acupuncture on HT8 after saline-injection

Scale bar = 100  $\mu$ m.

bilaterally, but in the results, it was expressed unilaterally, on condition that there were no significant differences between right and left part of PVN.

### 6. Data analysis

To compare the number of c-Fos-positive cells in the PVN of each groups, results were expressed as a mean $\pm$ SEM and analyzed by analysis of variance (ANOVA) followed by LSD test. Significant differences were considered for  $P < 0.05$  values.

### III. Results

In animals injected with saline solution, a few numbers of c-Fos-positive cells

were observed in the PVN of hypothalamus 4 hrs after injection (Fig. 1A). The LPS-injected group showed a strong increase in the number of c-Fos-positive cells compared to the saline-injected group at the same concordance points (Fig. 1B). However, this increase of c-Fos-positive cells in the PVN was counteracted by acupuncture on HT8 in LPS-injected rats (Fig. 1C). Acupuncture on ST36 did not alter the increased number of c-Fos-positive cells in the PVN in LPS-injected rats (Fig. 1D). The acupuncture treated- group (HT8) following saline-injection did not change the number of c-Fos-positive cells in the PVN (Fig. 1E).

The number of c-Fos-positive cells in the right side of PVN was noted because there was no significant difference between right and left part of PVN. The number of c-Fos-positive cells in the PVN per section was  $29.00 \pm 5.85$  (mean $\pm$ SEM) in the saline-injected group. The number of PVN c-Fos-positive cells in the LPS-injected group was  $203.00 \pm 41.24$ , which was significantly higher than that of the saline-injected group. The number of c-Fos-positive cells in the PVN of LPS-injected animals with acupuncture on HT8 was  $52.83 \pm 11.95$ , which was significantly decreased compared to the LPS-injected group. The number of PVN c-Fos-positive cells, in LPS-injection group with acupuncture on ST36 and saline-injection group with acupuncture on HT8, were reported to values of  $151.50 \pm 35.43$ ,  $38.17 \pm 8.93$  respectively (Table 1).

The number of c-Fos-positive cells in the PVN was significantly increased by LPS-injection and decreased after acupuncture on HT8, but not on ST36 (Fig. 2). These results indicate that acupuncture on HT8

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Table 1. The number of c-Fos positive neurons in the PVN of hypothalamus (mean  $\pm$  SEM)

Group	Animal	The number of c-Fos-positive cells
Saline	6	29.00 $\pm$ 5.85
LPS	6	203.00 $\pm$ 41.24
LPS+HT8	6	52.83 $\pm$ 11.95
LPS+ST36	6	151.50 $\pm$ 35.43
Saline+HT8	6	38.17 $\pm$ 8.93

inhibits the LPS-induced neuronal activation in the PVN of rats.

#### IV. Discussion

In this study, LPS injection (400  $\mu$ g/kg, i.v.) showed a similar time-course distribution of neuronal activation in the PVN to that described previously<sup>9,21,37</sup>. It is suggested that neurons in the PVN, stimulated after LPS-injection, may activate the HPA axis to cause the systemic symptoms of animals in this experiment.

According to the results, neurons in the PVN were activated 4 hrs after LPS-injection, while these activation were significantly attenuated by acupuncture on HT8. In saline-injected rats, however, acupuncture on HT8 did not change the neuronal activation of PVN. These results suggest that acupuncture on HT8 may suppress the activation of HPA axis and consequently reduce the infection-like symptoms, e.g. fever in LPS-injected rats. In Oriental medicine, the acupoint HT8 is well known to express the Hyung Points (榮穴) among the Five Su Points (五輸穴), and has been believed to be effective in treating fever-related disorders from immune dysfunction<sup>1,31</sup>. This effect of HT8 was clarified in the LPS-induced

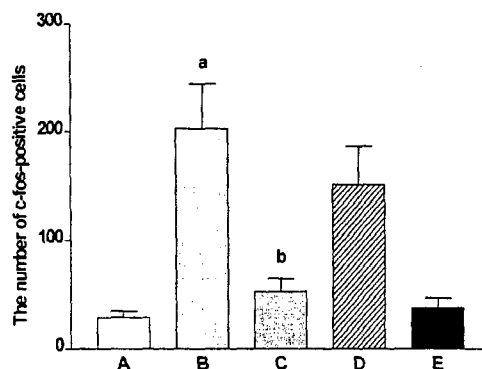


Fig 2. The number of c-Fos-positive cells in the paraventricular nucleus of hypothalamus 4 hrs after injection.

The number of c-Fos-positive cells was significantly increased by LPS-injection (B; <sup>a</sup>  $P < 0.001$ ) compared to saline-injected group (A). However, it was strongly attenuated by acupuncture on HT8 (C; <sup>b</sup>  $P < 0.001$ ), but not on ST36 (D). Acupuncture on HT8 showed no significant changes on the number of c-Fos-positive cells of saline-injected rats (E). Values were represented as mean  $\pm$  SEM (bar). Differences among groups were analyzed using ANOVA followed by *post-hoc* LSD test.

infection model via the method of c-Fos immunohistochemistry in this study.

Acupuncture on ST36 slightly suppressed an increase of neuronal activation in the PVN of LPS-injected rats, but not to a significant value. Although ST36 has been used for the treatment of various diseases, e.g. pain, gastric disorder and stroke<sup>16,19,20</sup>, it is not effective for infectious condition. It may also demonstrate the fact that each acupoints shows a unique and diverse effect for the purpose of treating diseases.

The expression of the proto-oncogene c-Fos has been known as a useful marker for neuronal activation, and its expression in the

various brain regions containing PVN after mild and acute physical stress has been reported<sup>14,29</sup>. C-Fos expression in the PVN after saline-injection might be caused by the stress from light immobilization and injection during the handling process. Recent report suggests that restraint and LPS-injection showed similar effect on c-Fos expression in the PVN with regard to the intensity and their distribution pattern<sup>38</sup>. Thus, significant difference in c-Fos immunoreactivity in the PVN between saline- and LPS-injected groups indicates the obvious induction of c-Fos expression by LPS.

Many c-Fos studies have been conducted for the investigation of analgesia effect of electroacupuncture (EA). These studies reported that brain stem, hypothalamus and spinal cord showed regional increases of c-Fos immunoreactivity after EA-induced analgesia<sup>22,24</sup>. C-Fos immunohistochemistry has been also used to investigate the therapeutic effects of acupuncture. C-Fos immunoreactive cells were increased in the spinal cord after noxious stimulation and decreased by acupuncture<sup>4</sup>. Acupuncture suppressed the c-Fos expression evoked by tooth pulp stimulation in the trigeminal subnucleus pars caudalis and the periaqueductal gray of rats<sup>28</sup>.

In the present study, c-Fos immunoreactivity was used for the evaluation of neuronal activation to examine the acupuncture-induced modulation on the process of inflammatory response in the PVN.

Previous reports showed the effects of acupuncture on peripheral immune system in normal or pathological conditions<sup>6,11,23, 25</sup>. Recently, it was reported that LPS-induced

cytokine mRNAs in the hypothalamus were reduced by acupuncture treatment with the attenuation of bacterial fever in rats<sup>30</sup>. The present immunohistochemical study was conducted in the same way with previous studies to prove the potent actions of acupuncture on the immune system, but we paid focus on specific area of rat brain, the PVN.

In conclusion, this study provided an evidence that stimulation on specific acupoint such as HT8 may exert anti-inflammatory effect by down-regulating the expression of immediate early genes of cytokine in PVN hypothalamus of LPS inflammation rat model.

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