

소염방 약침이 흰쥐 시상하부 면역 조절에 미치는 영향

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The Effects of Herbal Acupuncture on Immuno-regulatory Action of the Hypothalamus in Rats

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Abstract

목적: 본 연구는 염증유발 내독소인 LPS 자극을 통한 면역 스트레스에 대한 약침의 치료효과와 기전을 알아보기 위해 설계되었다. LPS를 통한 염증반응은 다양한 기전을 통해 이루어지는 데 특히, 산화질소(NO)를 생성하는 효소인 iNOS의 활성화는 시상하부에서 NO의 생성을 촉진함으로써 염증반응을 발현시키는 중요한 역할을 하는데 약침이 LPS로 인해 활성화된 iNOS의 발현에 어떠한 영향을 미치는지 알아보고자 하였다.

실험방법: 흰쥐에 LPS를 투여하고 2시간 경과한 후 약침(소염방, 대한약침학회)을 경혈(합곡, 족삼리)에 좌,우 각각 0.2cc 씩 피하 투여하고 RT-PCR 방법을 통하여 면역활성에 중요한 역할을 하는 시상하부에서의 iNOS의 mRNA 발현을 알아 보았다.

실험결과: LPS 투여로 인하여 증가하였던 iNOS mRNA가 약침치료를 통해 현저하게 감소하였다. 그러나 비경혈점(미추부위 임의혈)에 약침을 투여한 군과 경혈점에 생리식염수를 투여한 군에서는 iNOS mRNA가 감소하지 않았다. 이는 약침이 LPS를 투여한 흰쥐의 시상하부에서 iNOS의 mRNA 발현에 조절 작용을 가지고 있는 것을 보여주는 결과로 약침이 면역활성에 중요한 역할을 하는 NO의 생성을 억제함으로써 면역 스트레스에 치료효과를 나타내는 것으로 보여진다.

결론: 본 연구는 약침치료가 면역반응의 중추기관인 시상하부에서 면역반응의 중요한 조절인자인 NO의 생성을 조절하는 기전을 통해 그 치료효과를 나타내는 것을 밝혔고 더 나아가 약침에 대한 현대과학적 연구에 중요한 발판을 마련했다는 점에서 그 의의를 가지고 있다고 사료된다.

Key words: 약침, Lipopolysaccharide, RT-PCR, inducible nitric oxide synthase, 시상하부.

I. Introduction

The herbal acupuncture therapy is a new way of stimulating acupoints, which is done by injecting herbal extraction to the acupoints, and it has been frequently used

for immune regulation in clinical settings^{2),14)}.

Although there has been several experiments to provide scientific evidences on the mechanism of herbal acupuncture therapy^{3,25)}, it is yet largely unknown.

The antipyretic effects of acupuncture stimulation have been vigorously studied in recent years. The effects of acupuncture

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stimulation on the Gokji (LI11) and Hapgok (LI4) loci on both thermoregulatory function and analgesic activity were assessed in normal adults^{1,23}. The Gokji (LI11) and Hapgok (LI4) have been reported to have the effects of thermoregulatory function and analgesic activity in normal adults^{4,10}. The effects of acupuncture stimulation on the Daechu (GV14), Naeguan (EH6) and Joksamli (ST36) loci on thermoregulatory function were studied in normal adults¹⁵. Electro-acupuncture (EA) stimulation to the Gokji (LI11) has antipyretic effects on fever induced by either lipopolysaccharide (LPS), interleukin-1 beta (IL-1 beta) or prostaglandin E2 (PGE2). EA stimulation possesses an antipyretic effect through the inhibition of the action of PGE2 in rats⁵. EA stimulation to Daechu (GV14) has an inhibitory effect on arthritis, and the partial mechanism of its therapeutic result may be attributed to the inhibition of the production of IL-1 beta and PGE2 by suppressing the IL-1 beta and cyclooxygenase-2 gene activation⁶. Additionally, the acupunctural effects suggest participation of an endogenous opioid system in initiation of a number of responses mediating the antipyretic and immuno-regulatory effect of acupuncture²⁰.

LPS is an endotoxin derived from cell wall of gram-negative bacteria. LPS effect occurs 2 hours after the injection by stimulating cytokines to induce acute phase response (APR). LPS induces mRNA

synthesis for cytokines (IL-1, IL-6, tumor necrosis factor-alpha) in microglial cells. LPS induces significant increase in nitric oxide synthase (NOS) gene expressions of the paraventricular nucleus (PVN) with different time-course and distribution⁸. Nitric oxide (NO) generation, especially by inducible NOS (iNOS) in rat's hypothalamus and pituitary, is involved in the mechanism of LPS-induced fever generation²¹.

The present study is designed to investigate the immuno-regulatory effect and its mechanism of herbal acupuncture on inflammatory response in LPS-injected rats. We observed the mRNA expressions of iNOS in the hypothalamus using the RT-PCR method.

II. Material and Method

1. Animal

Male Sprague-Dawley rats weighing 250 ± 20 g were used in this experiment. All animals were supplied from Korean Experimental Animal Center and treated according to NIH guideline.

2. material

(1) Lipopolysaccharide (LPS; E. coli, 055:B5, Sigma, USA) was used for animal infection model. For herbal acupuncture treatment, the drug was provided by Korean Institute of Herbal Acupuncture for the experimental use.

(2) Ingredients of drug

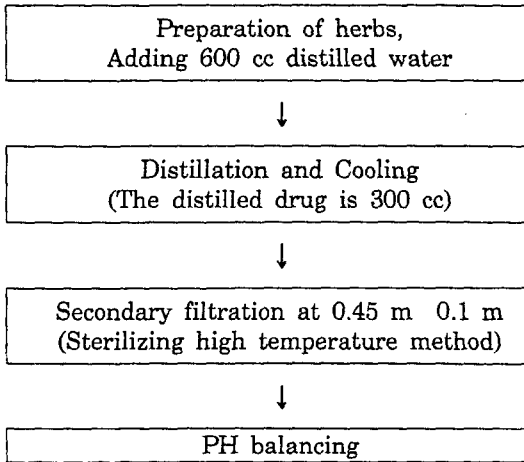


Fig. 1 Methods of extracting herbal acupuncture drug.

'SY' is a drug extracted from herbal complex. The herbal complexes were made up of Coptidis Rhizoma 24 g, Scutellariae Radix 24 g, Phellodendri Cortex 24 g, Gardeniae Fructus 24 g, Loniceræ Flos 39 g, Forsythiae Fructus 39 g, Taraxaci Herb 39 g and Rehmaniae Radix (Table 1). The method of making drug was shown in Fig.1¹¹⁾.

3. LPS Injection

LPS (400 g/kg, i.v.) diluted in normal saline was injected to induce LPS infection model. Normal sterilized saline was injected by same method for saline control group.

4. Herbal acupuncture treatment

Two acupuncture points - Hapgek (LI 4) and Joksamli (ST36) - were selected for herbal acupuncture treatment. Hapgek (LI 4) is on the dorsal side of the forepaw, between

Table 1. The prescription of SY and its function

Drug	Function
Coptidis Rhizoma (berberine)	1. Clearing away heat and eliminating dampness. 2. Clearing away heart-fire to relieve fidgetiness. 3. Purging pathogenic fire and lessening.
Scutellariae Radix	1. Eliminating heat and resolving dampness. 2. Purging fire and removing toxic materials. 3. Eliminating heat and calming fetus.
Phellodendri Cortex	1. Reducing heat and resolving dampness. 2. Purging fire and eliminating virulent evil factors. 3. Eliminating asthenia-heat.
Gardeniae Fructus	1. Purge sthenic fire to relieve vexation. 2. Clear away heat and promote diuresis. 3. Clear away heat and toxic materials. 4. Cool the blood and stop bleeding for heat-syndrome
Loniceræ Flos	1. Removing heat and toxic substance. 2. Removing heat from the blood and arresting dysentery.
Forsythiae Fructus	1. Reducing intense heat and eliminating virulent substances 2. Eliminating fire and dispersing mass
Taraxaci Herba	1. Reducing intense heat and eliminating heat toxics. 2. Clearing away liver fire and improving vision
Rehmaniae Radix	1. Clear away heat and cool the blood, nourish yin to promote the production of body fluid

1st and 2nd metacarpal bones. Joksamli (ST36) is located 5 mm laterally and distally from the anterior tubercle of the tibia. For treatment, 0.1 ml of herbal acupuncture

solution ('SY' Research Center of KIHA, Korea) was bilaterally injected into each point. Same amount of solution was injected at non-acupoint located at proximal tail. All treatments were performed 2 hours after LPS or saline injection.

5. RT-PCR

Four hours after injection, the animals were sacrificed and their brains were removed. The hypothalamus was isolated as quickly as possible and was immediately homogenized in 0.5 ml of pre-cooled Trizol reagent (Gibco BRL). The total RNA was extracted according to the manufacturer's instruction and then subjected to electrophoresis in 1% agarose gel for confirmation of the structural integrity. The absorbance at 260 nm was measured for estimation of the RNA concentration. Three 1 of total RNA was reverse transcribed (RT) with oligo-dT primers by using MMLV reverse transcriptase (Promega) in a total volume of 12 1. PCRs were performed in 50 1 reaction volume containing 10 mM dNTP 0.8 1, 2.5 unit Taq polymerase (Promega), 10×buffer 5 1, 20 pmol of primer pairs and 2 1 cDNA. The amplification was then performed in a thermo-cycler (Corebio system, Germany), beginning with a 5 min pre-incubation at 94 °C, followed by 35 cycles of 45 s at 94°C, 60 s at 58°C and 80 s at 72°C, ending with a 5 min incubation at 72°C. The amplified products were electrophoresed on 1.5% agarose gel, visualized with ethidium bromide for fragment size estimation. For quantifica-

Table 2. The sequences of primers and fragment sizes

cDNA		Sequence of primer	Fragment size (bp)
iNOS	sense	5'-ACAACAGGAACC TACCAGCTCA-3'	651
	anti-sens	5'-GATGTTGTAGCG CTGTGTGTCA-3	
GAP DH	sense	5'-TCCCTCAAGATT GTCAGCAA-3'	309
	anti-sens	5'-AGATCCACAACG GATACATT-3	

tion of band intensities, the images were scanned and analyzed by gel analysis software (BIO-RAD).

6. Data analysis

With RT-PCR data, to correct subtle sample-to-sample differences in the amounts of starting material, all values were normalized to GAPDH herbal acupuncture treatment served as internal control. Differences between groups were analyzed using student *t*-test or one-way analysis of variance (ANOVA) followed by *post-hoc* LSD test for multiple comparisons if needed. *P* < 0.05 was considered as the level of significance.

III. Result

In hypothalamus, iNOS mRNA expression was significantly increased 4hrs after LPS-injection compared to herbal acupuncture treatment in saline-injected rats (Fig. 2). However, herbal acupuncture treat-

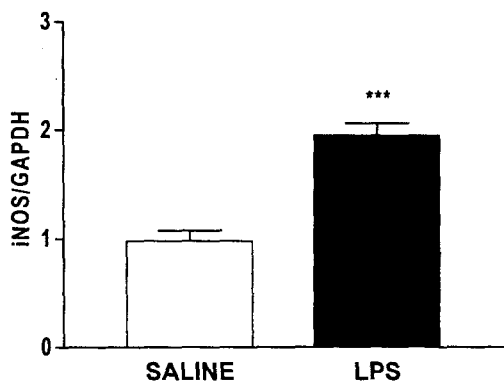


Fig. 2 iNOS mRNA expression in the hypothalamus was significantly increased by LPS injection. (***) ; $P < 0.001$, vs. saline group, student *t*-test) implying the effective induction of acute inflammatory and immune-activated condition through the peripheral endotoxin.

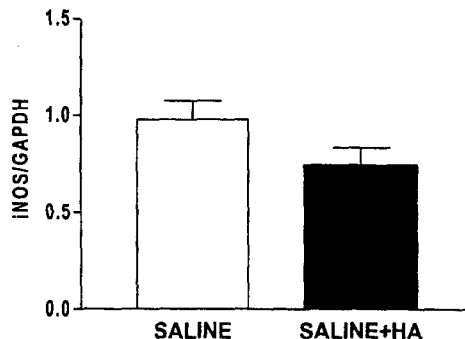


Fig. 4 Herbal acupuncture at LI4 and ST36 has no significant effect on the iNOS mRNA expression in the hypothalamus of saline-injected rats.

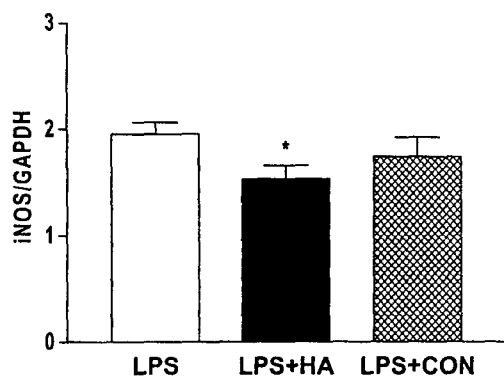


Fig. 3 Herbal acupuncture at LI4 and ST36 (LPS+HA) significantly reduced the iNOS mRNA expression that increased by LPS-injection in the hypothalamus (*; $P < 0.05$, vs LPS group, student *t*-test), but the same treatment at non-acupoint (LPS+CON(control)) had no significant effect. Fig. 4. Distribution of NPY immunoreactive neurons in PVN. (A) Normal (B) maternally separated group (C) HT7 group with maternal separation. Scale bar: 250 μ m.

ment significantly suppressed the iNOS mRNA expression that was increased by LPS-injection in the hypothalamus. Control treatment did not decrease it in the LPS-injected rats (Fig. 3). Herbal acupuncture treatment did not change the iNOS mRNA expression in the saline-injected rats (Fig. 4).

The levels of iNOS mRNA normalized to GAPDH of each groups were as following saline-injected group, 0.98 ± 0.10 (mean \pm S.E.M); LPS-injected group, 1.95 ± 0.11 ; herbal acupuncture treatment after LPS-injection group, 1.53 ± 0.12 ; control treatment after LPS-injection group, 1.73 ± 0.19 ; herbal acupuncture treatment after saline-injection group, 0.75 ± 0.09 .

IV. Discussion

LPS is an endotoxin derived from cell wall of gram negative bacteria. Systemic injection of LPS results in many symptoms of bacterial infection including fever and inflammation¹⁵⁾. LPS makes profound changes

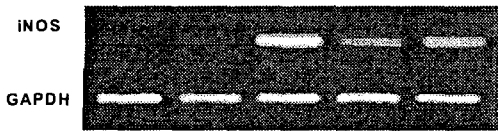


Fig. 5 iNOS mRNA expression was strongly increased by LPS (LPS), but it was effectively attenuated by herbal acupuncture treatment at LI4 and ST36 (LPS+HA), not by the same treatment at non-acupoint (LPS+C). Herbal acupuncture at LI4 and ST36 (S+HA) has no significant effect on the iNOS mRNA expression in the hypothalamus of saline-injected rats (S).

S : Group with saline-injection

S+HA : Group with herbal acupuncture after saline-injection

LPS : Group with LPS-injection

LPS+HA : Group with herbal acupuncture after LPS-injection

LPS+C : Group with control treatment after LPS-injection

in monoamine concentrations in specific areas of the hypothalamus including the PVN, which have been implicated in fever production and hypothalamic pituitary adrenocortical activation²⁴. LPS and cytokines induce NO synthesis and NO regulates the synthesis and releasing of cytokines¹⁰.

NO is a molecule involved in different homeostatic control mechanisms including the temperature regulation. NO is generated from amino acid L-arginine by oxidative deamination, and this synthesis is catalyzed by NOS¹⁸. NOS has three isoforms: type I or neuronal NOS (nNOS), which is implicated in neuronal signaling, type II or an inducible isoform (iNOS), which is synthesized by macrophages in response to inflammatory stimuli, and type III or a constitutive isoform,

endothelial NOS (eNOS), which is principally present in the endothelium and play a substantial role in blood pressure control¹⁹. iNOS inhibits further release of cytokines by immune cells and plays a role in the mechanism of pyrogen signal transduction at the blood-brain barrier⁹. NO generation, especially by iNOS in the rat hypothalamus and pituitary, is involved in the mechanism of LPS-induced fever generation²¹.

In this study, injection of 'SY'drug at Hapgok (LI4) and Joksamli (ST36) points significantly attenuated iNOS mRNA expression in the hypothalamus of rats. On the contrary, control non-acupoint treatment group did not decrease iNOS mRNA expression in the LPS-injected rats, and herbal acupuncture treatment after saline-injection group did not change it.

Herbal acupuncture is a new therapeutic method of the Oriental medicine, which utilize the injection of the extracts from herbal complex into the acupoints to obtain the effects of acupuncture and herbal drug^{2,11,17,22}. 'SY' is used frequently for antipyretic and immuno-regulatory treatment in herbal acupuncture therapy. It consists of Coptidis Rhizoma, Scutellariae Radix, Phellodendri Cortex, Gardeniae Fructus, Lonicerae Flos, Forsythiae Fructus, Taraxaci Herba, and Rehmanniae Radix¹⁶. These herbs are usually used for fever-related disorders or immune-dysfunction.

Two acupuncture points, Hapgok (LI4) and Joksamli (ST36), were selected for

herbal acupuncture treatment in this study. The function of Hapgok (LI4) is clearing and discharging lung heat. It is 'Won'(primary) point of the large intestine meridian and command point of the face. The indications of Hapgok (LI4) are headache, pain in the neck and eye, facial paralysis etc. The function of Joksamli (ST36) is freeing and regulating 'Qi' and blood of the channel. It is 'Hap' (earth) point of the stomach meridian and command point of the stomach. The indications of Joksamli (ST36) are gastric pain, vomiting, abdominal distension, aching of knee joint etc.

V. Conclusion

The results showed that LPS induced the increase of iNOS mRNA levels in the hypothalamus, which suggested systemic inflammatory response. However, mRNA levels of these pro-inflammatory cytokines in the hypothalamus were significantly decreased by herbal acupuncture treatment. It might be explained by inhibitory action of herbal acupuncture on endotoxin-induced pathologic inflammatory response in the hypothalamus.

Therefore, the present results suggested that immuno-regulatory action of herbal acupuncture might be accomplished partially by the suppression of hypothalamic gene expression of iNOS in LPS-injected rats.

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