

Original Article

Anti-inflammatory Effect of Bee Venom Acupuncture at *Sinsu*(BL₂₃) in a MPTP Mouse Model of Parkinson Disease

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국문초록

MPTP 유발 파킨슨 병 동물 모델에서의 신수혈(BL₂₃) 봉독약침의 항염증 효과

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목적 : 파킨슨 병은 기저핵 흑질의 치밀부에서 도파민성 신경세포의 퇴행으로 인하여 발생하는 질병으로 신경 염증이 주요 병인으로 밝혀져 있다. 이 연구는 MPTP 유발 파킨슨 병 동물 모델에서 신수혈(BL₂₃)에 대한 봉독 약침의 항염증 효과 및 그 기전을 확인하기 위해 시행되었다.

방법 : C57BL/6쥐를 무처치군, MPTP+saline군, MPTP+BVA(0.06mg/kg)군, MPTP+BVA(0.6mg/kg)군의 4군으로 나눈 뒤 무처치군을 제외한 모든 그룹에 총 8시간 동안 2시간 간격으로 MPTP-HCl(20mg/kg per dose×4)을 복강내로 주입하였다. MPTP+BVA 군에서 봉독약침은 마지막 MPTP 주입 2시간 후부터 48시간 간격으로 신수혈(BL₂₃)에 양측으로 각 20 μ l씩 주입하였고 MPTP+saline군에서는 봉독약침 대신 Saline을 주입하였다. 마지막 MPTP 주입 후 7일째에 쥐의 뇌를 적출한 후 면역조직화학법을 시행하였다.

결과 : MPTP 유발 파킨슨 병 동물 모델에서 신수혈에 대한 봉독약침은 농도 의존적으로 TH-Immunoreactivity neuron의 감소와 microglial activation을 억제하였다. HSP70-IR neuron은 모든 군에서 나타나지 않았다.

결론 : 봉독약침이 용량 의존적으로 microglial activation을 억제하는 효과를 통해 도파민성 신경세포의 파괴를 억제함으로써 항염 효과를 나타냄을 알 수 있었다. 이 결과는 봉독약침이 microglial activation 억제를 통해 임상적으로 파킨슨 병과 같은 신경 퇴행성 질병에 있어 유용한 치료수단이 될 수 있음을 시사한다.

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핵심단어 : Bee venom, Acupuncture, Parkinson disease, MPTP (*1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*), *Sinsu*(BL₂₃: *Shen Shu*), Heat shock protein(HSP)

I. Introduction

Parkinson disease(PD) is one of the most common movement disorders. The cardinal symptoms of the disease include a paucity of spontaneous movement, akinesia, bradykinesia, rigidity, and a characteristic tremor at rest. In the early 1960s, PD was shown to result largely from the degeneration of dopaminergic neurons in the substantia nigra pars compacta(SNpc)¹⁾. Brain inflammation is the common final pathway in PD²⁾. Central to this inflammation is the activation of microglia, which act as intrinsic immune effectors when the brain is injured^{3,4)}. Microglia are known to be activated in the PD-affected brain, and MPTP-induced microglial activation is associated with oxidative stress⁵⁾.

Bee venom contains a variety of peptides and proteins with a range of molecular weights, including melittin, apamin, adolapin, mast cell degranulating peptide and phospholipase A₂⁶⁻⁸⁾. In addition, it also contains biologically active amines(histamine, epinephrine) and several other non-peptide components including lipids, carbohydrates and free amino acids⁹⁾. Bee venom(BV) acupuncture(BVA) has been traditionally used in Oriental medicine to relieve pain and to treat chronic inflammatory diseases such as rheumatoid arthritis¹⁰⁾, and neurodegenerative diseases such as multiple sclerosis^{11,12)}. Recent studies have suggested that BV has anti-inflammatory properties that inhibit the production of inflammatory cytokines and nitric oxide(NO) in neurodegenerative diseases^{13,14)}.

Several animal models of PD have been developed, including a mouse model in which a parkinsonian pathology develops in response to the administration of the neurotoxin *1-methyl-4-phenyl-1,2,3,6-*

tetrahydropyridine(MPTP). MPTP causes many of the hallmark features of PD in humans and nonhuman primates, and it induces SNpc-specific dopaminergic cell loss in other mammalian species including mice¹⁵⁾.

The heat shock proteins(HSPs) are so named due to the fact that their synthesis was initially found to be enhanced in response to an increase in temperature¹⁶⁾. HSPs represent an important cellular protective mechanism against a variety of stresses and insults¹⁷⁻¹⁹⁾. Recent studies have suggested that HSP may be involved in neuronal cell death in PD^{20,21)} and may play a neuroprotective role in MPTP-induced neurotoxicity²²⁾. Thus, HSPs may represent an important molecular target for neuroprotective strategies in PD treatment, and geldanamycin is known to induce the expression of HSP70 and protect against MPTP-induced dopaminergic neurotoxicity in mice²³⁾.

In this study, we used an MPTP-induced neuroinflammation mouse model to examine whether if (a) BVA inhibits the loss of tyrosine hydroxylase (TH)-positive neurons as a result of its inhibition of microglial activation or induction of HSP70 synthesis and (b) the effect of BVA is dose dependent. Microglial activation was measured by the level of macrophage antigen complex(MAC-1) expression.

II. Materials and Methods

1. Animals and MPTP administration

Six-week old male C57BL/6 mice(Samtaco Co., Korea), weighing 20–25g, were used in all experiments.

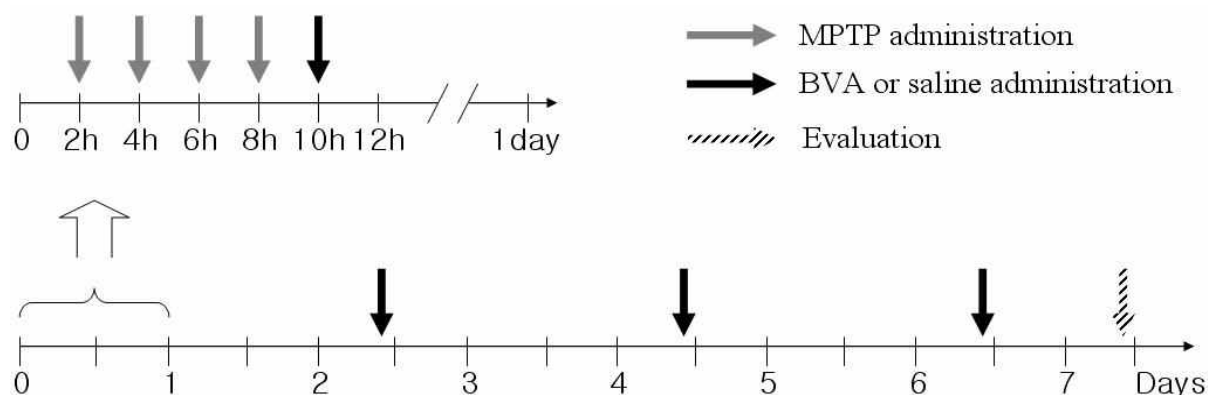


Fig. 1. MPTP & BVA administration schedule

At the beginning of the experiment, the animals were randomly divided into four groups: a naïve group, an MPTP+saline group, an MPTP+BVA(0.06mg/kg) group and an MPTP+BVA(0.6mg/kg) group. All mice except those in the naïve group received an intraperitoneal(i.p.) injection of MPTP-HCl(20mg/kg per dose \times 4; Sigma, St. Louis, MO, USA) in saline at 2h intervals over an 8h period in 1day.

In the MPTP+BVA groups, BVA administered at BL₂₃(bilateral; 20 μ l per point) began 2h after the last MPTP injection and then resumed at 48h. In the MPTP+saline group, saline was injected instead of BV. All mice were sacrificed seven days after the last MPTP injection. Mice in the naïve group were sacrificed on the same day.

The mice were acclimated for 2 weeks in cages at 21°C and were provided with water and food ad libitum. At the beginning of the experiment, the animals were randomly divided into four groups: A naïve group, an MPTP+saline group, an MPTP+BVA(0.06mg/kg) group and an MPTP+BVA(0.6mg/kg) group. Animal experiments were carried out in accordance with the National Institute of Health's Guide for Care and Use of Laboratory Animals, and experimental procedures were approved by the Institutional Animal Care and Use Committee at the Kyunghee University. Except for the naïve group, the mice(six per group) in the other groups received an intraperitoneal(i.p.) injection of MPTP-HCl(20mg/kg per dose \times 4; Sigma, St. Louis, MO, USA) in saline every 2h, over an 8h period, in 1 day²⁴). For i.p. injection, MPTP was dissolved in 5 μ l saline and an i.p. injection was performed as previously described²⁴) using a 30- μ l Hamilton syringe with a 30-gauge needle. The animals were sacrificed at seven days after the last MPTP injection(Fig. 1).

2. Treatment with diluted bee venom

Bee venom (Sigma, St. Louis, MO, USA) was diluted to doses of 0.06mg/kg in 40 μ l of saline and

0.6mg/kg in 40 μ l of saline; 20 μ l of each dose were subcutaneously administered bilaterally into each BL₂₃ point, located at the depression lateral to the lower border of spinous process of the second lumbar vertebra²⁵). Thus, total injection volume of 40 μ l was administered to each subject, as described previously²⁶).

BVA at BL₂₃ began 2h after the last MPTP i.p. injection and then resumed at 48h intervals until the mice were sacrificed, seven days after the last MPTP i.p. injection. For this procedure, the mice in the MPTP+BVA groups were immobilized, and BV was administered into BL₂₃. For the MPTP+saline group, 40 μ l of saline was injected in place of BV.

3. Tissue preparation and immunohistochemistry

The mice in all groups were sacrificed by anesthesia with pentobarbital sodium (60mg/kg, i.p.) 7days after the last MPTP i.p. injection and perfused transcardially with paraformaldehyde (4% in 0.1M phosphate buffer, pH 7.4). The brains were isolated, post-fixed in the same fixative overnight, subsequently cryoprotected with 30% sucrose in 0.05M phosphate-buffered saline(PBS, pH 7.4) for

48h, and sectioned coronally into 30µm slices for histological analysis.

For the immunohistochemistry, brain sections were incubated with one of the following antibodies: (1) rabbit anti-tyrosine hydroxylase(TH) antibody (Chemicon; 1 : 4,000), (2) rabbit anti-MCA-1 antibody (Serotec; 1 : 500) and (3) rabbit anti-heat shock protein (HSP70) antibody (Chemicon; 1 : 2,000). Brain sections were treated with primary antibody at room temperature for 16h, followed by biotinylated secondary antibody (1 : 200; Vector) for 1h. Then the sections were incubated with ABC solution (1 : 100; Vector) and finally developed in DAB or Ni-DAB solution. Sections were dehydrated with alcohol and xylene, and then mounted with Permount solution.

4. Quantitative analysis

SNpc neuronal counts were manually performed by technicians who were blinded to the treatment schedule. TH-IR cells in the SNpc were bilaterally counted using a confocal microscope (Multiscan, Fullerton, CA, USA) for at least 3 TH-immunostained mesencephalic sections at the widest dimension of the SNpc at AP-3.16²⁷⁾, lateral to the roots of the third cranial nerve separating the medial and lateral SNpc. Evaluation of the neuronal staining intensity was performed by measuring the optical density of MAC-1-IR and HSP70-IR neurons in 10 sections from the substantia nigra. The optical density of the stained neurons was quantitatively assessed by microdensitometry using an image analyzer(Multiscan, Fullerton, CA, USA). Before the densitometry measurement, the voltage-related change in optical density was evaluated. The optimal voltage was then obtained from the linear portion of the S-shaped voltage-related optical density curve²⁸⁾. During full measurement of the optical density, the optical voltage was maintained at a constant level.

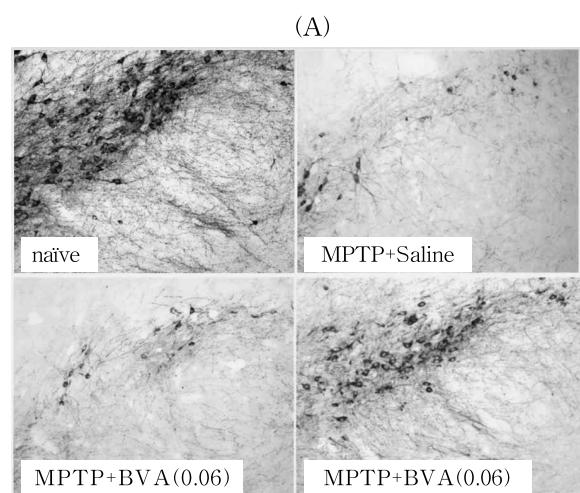
5. Statistical analysis

Means and standard deviation(SD) were calculated for the estimated numbers of TH-IR neurons and the optical density of MAC-1-IR and HSP70-IR positive neurons. To rule out a possible change in SNpc volume as an influencing factor, the number of TH-IR neurons are expressed as a ratio of normal controls per area of SNpc. Statistical analyses were performed using analysis of variance (ANOVA). A Bonferroni Multiple Comparison Test was used to compare individual means. Differences between the means of experimental groups were considered significant at $p < 0.05$.

III. Results

1. BVA inhibits the MPTP-induced neuronal loss of TH-IR neurons in the SNpc in a dose dependent manner

Immunohistochemical staining with TH antibody was performed on the brain samples collected from each group 7days after the last MPTP injection. TH-IR neurons were counted bilaterally at least three TH-immunostained mesencephalic sections. TH-IR neurons were plentiful in the SN of the



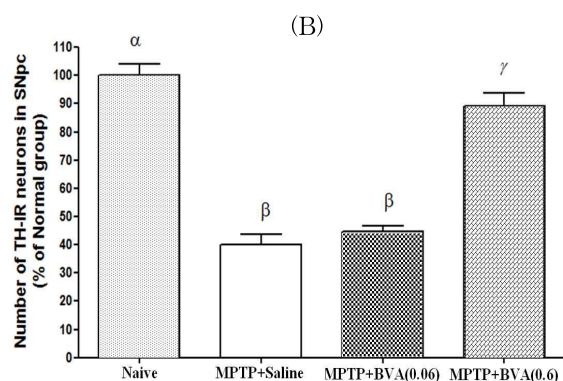


Fig. 2. Effects of BVA at BL₂₃ on MPTP-induced loss of tyrosine hydroxylase (TH)-immunoreactive (IR) neurons in the substantia nigra pars compacta (SNpc)

(A) shows photomicrographs of TH-IR neurons of the SNpc of MPTP-induced C57BL/6 mice 7 days after the last injection of MPTP. Naive, naïve mice; MPTP+saline, mice treated with bilateral saline acupuncture at BL₂₃ after MPTP administration; MPTP+BVA(0.6), mice treated with bilateral BVA at BL₂₃(0.6mg/kg) after MPTP administration; MPTP+BVA(0.06), mice treated with bilateral BVA at BL₂₃(0.06mg/kg) after MPTP administration.

(B) shows levels of the number of TH-IR neurons in the SNpc 7 days after the last injection of MPTP. The levels are expressed as the ratio of the average number of TH-IR neurons per section in the naïve group. All values represent means \pm S.D. of the average number of TH-IR neurons per section in the naïve group(n=6). Means designated by the same letter are not significantly different according to the Bonferroni Multiple Comparison test($\alpha=0.05$).

naïve group(Fig. 2A).

The survival ratios of TH-IR neurons in the SNpc of the MPTP+saline and MPTP+BVA(0.06) groups were only 40% and 44%, compared with the naïve group. In contrast, in the MPTP+BVA(0.6) group, the survival ratio of TH-IR neurons was 89% which was significantly different from the survival ratios of the MPTP+saline and MPTP+BVA(0.06) groups(Fig. 2B).

2. BVA at BL₂₃ inhibits the MPTP-induced activation of MAC-1-IR neurons in the SNpc in a dose dependent manner

The SNpc is relatively rich in microglia compared with other brain regions^{29,30}. Previous studies have suggested that activation of microglia

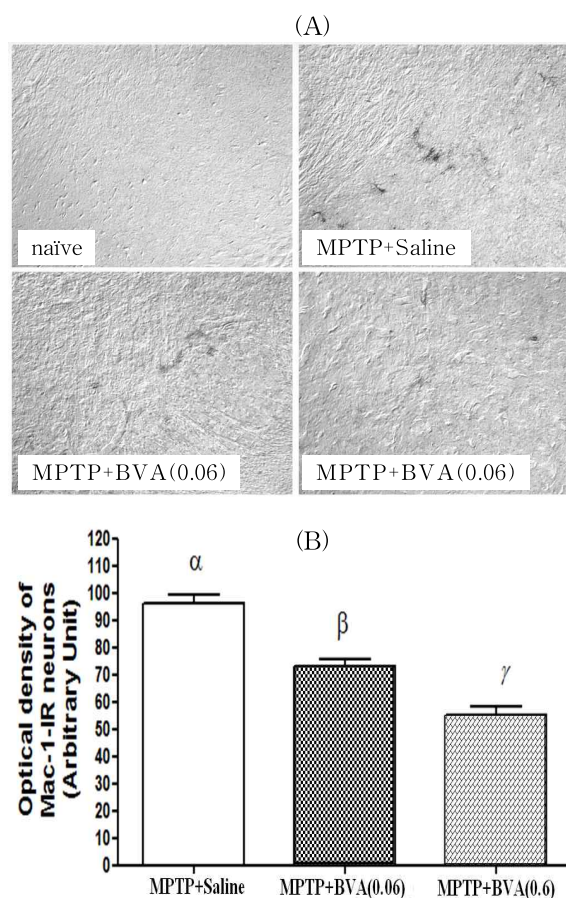


Fig. 3. Effects of BVA at BL₂₃ on MPTP-induced microglial activation of MAC-1-IR neurons in the SNpc

(A) shows photomicrographs of MAC-1-IR neurons of the SNpc of MPTP-induced C57BL/6 mice on 7 days after the last MPTP injection. Naïve, naïve mice; MPTP+saline, mice treated with bilateral saline acupuncture at BL₂₃ after MPTP administration; MPTP+BVA(0.6), mice treated with bilateral BVA at BL₂₃(0.6mg/kg) after MPTP administration; MPTP+BVA(0.06), mice treated with bilateral BVA at BL₂₃ (0.06mg/kg) after MPTP administration.

(B) shows the optical density levels of MAC-1-IR neurons in the SNpc. Optical density was measured in six sections throughout the entire rostrocaudal extent of the SNpc. The levels are expressed as the average optical density of MAC-1-IR neurons per section. Data are expressed as mean \pm S.D. of the average optical density for each section. Means designated by different letters are significantly different according to the Bonferroni Multiple Comparison test($\alpha=0.05$).

may trigger or participate in the neurodegenerative processes in PD²). To determine whether the beneficial effect of BVA was associated with inhibition of the MPTP-induced glial response, we examined the expression of MAC-1, a marker of

microglial activation, 7days after last the MPTP injection. In the naïve group, activated microglia were not observed. In the MPTP+saline group, marked expression of MAC-1 and an increase in dendritic processes surrounding the MAC-1-IR neurons were observed. In contrast, the optical density and expression of activated microglia were significantly decreased in the MPTP+BVA groups in a dose dependent manner, compared with the MPTP+saline group(Fig. 3A). In particular, the activation of MAC-1-IR neurons was significantly decreased in the MPTP-BVA(0.6) group compared with the MPTP+BVA(0.06) group(Fig. 3B).

3. HSP70-IR neurons were not observed in the SNpc of any group

Heat shock proteins(HSPs) play a neuroprotective role in MPTP-induced neurotoxicity²². To determine whether the beneficial effect of BVA was associated with HSPs, we examined the expression of HSP70,

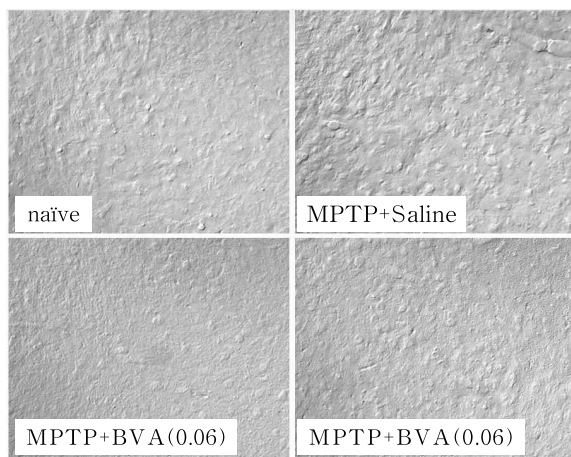


Fig. 4. Expression of HSP70-IR neurons of the SNpc of MPTP-induced C57BL/6 mice 7days after the last MPTP injection, and the relationship with BVA at BL₂₃

Photomicrographs of HSP70-IR neurons of the SNpc of MPTP-induced C57BL/6 mice, taken 7days after the last MPTP injection. Naïve, naïve mice; MPTP+saline, mice treated with bilateral saline acupuncture at BL₂₃ after MPTP administration; MPTP+BVA(0.6), mice treated with bilateral BVA at BL₂₃(0.6mg/kg) after MPTP administration; MPTP+BVA(0.06), mice treated with bilateral BVA at BL₂₃(0.06mg/kg) after MPTP administration. HSP70-IR neurons were not observed in the substantia nigra of any group.

7days after last MPTP injection. HSP70-IR cells were not observed in the substantia nigra of any group(Fig. 4).

IV. Discussion

Parkinson disease(PD), first described by James Parkinson in 1817, as paralysis agitans, or the 'shaking palsy', is the result of a quite specific and progressive neurodegeneration of pigmented nigro-striatal dopaminergic neurons. The symptoms of PD are only apparent after the loss of at least 50% of the dopaminergic neurons in the substantia nigra pars compacta(SNpc), which leads to a reduction of over 80% in the dopamine(DA) levels in the striatum^{31,32}.

The cause of PD remains unclear, but several theories have been proposed regarding the possible factors behind the neuronal degeneration. These include environmental toxins, genetic factors and mitochondrial dysfunction as well as free radical-mediated cell death and oxidative stress³³⁻³⁶. Recently, however, there has been increasing recognition of the possible major role of neuroinflammation in the pathogenesis of PD³⁷, induced by exposure to either infectious agents or toxicants with pro-inflammatory characteristics.

Microglia are resident immunocompetent and phagocytic cells in the central nervous system(CNS), and are thought to mediate the innate defense system and thus serve a critical role in normal CNS function³⁸. They are activated in the event of infection, inflammation, trauma, ischemia and neurodegeneration in the CNS³⁹. It has been shown that glial cells are activated in the PD brain and MPTP-induced glial activation in association with oxidative stress⁵. MPTP is a neurotoxin that induces Parkinsonian features in humans, rodents and non-human primates and has been demonstrated to cause rapid and selective DA neurotoxicity⁴⁰. Recent studies suggested that BV has anti-inflammatory properties that inhibit production of

inflammatory cytokines and NO in the activated microglia^{13,14}. Acupuncture also inhibits microglial activation and inflammatory events in the MPTP-induced mouse model⁴¹.

The heat shock proteins(HSPs) are so named due to the fact that their synthesis was initially found to be enhanced in response to an increase in temperature¹⁶. HSPs are a group of highly conserved stress proteins that play an important role in maintaining body self-stability. The main functions of HSPs are to promote cellular tolerance against stress factors, to maintain normal cellular physiological function, and to increase cellular defense and adaptation to deadly stimulation⁴². It is known that a plethora of insults other than heat stress have since been found to increase the expression of HSPs including neurotoxicants, drugs of abuse, environmental stress, metals, oxidative stress and many other pathophysiological states and non-stressful conditions. Previous data from in vivo and in vitro studies strongly suggest that HSPs play a neuroprotective role in MPTP-induced neurotoxicity²². Moxibustion and electroacupuncture can induce the synthesis of heat shock protein 70^{43,44}. However, to date no study had examined the relationship between BVA and HSPs.

In the present study, we used an MPTP-induced neuroinflammation mouse model to examine whether (a) BVA inhibits the loss of tyrosine hydroxylase(TH)-positive neurons as a result of its inhibition of microglial activation or induction of HSP70 and (b) the effect of BVA is dose dependent. Microglial activation was measured by the expression of MAC-1. Due to the strain-dependent sensitivity to MPTP⁴⁵, we used C57BL/6 mice.

TH is the rate-limiting enzyme in the synthesis of the catecholamine neurotransmitters, such as dopamine, epinephrine, and norepinephrine. It converts L-tyrosine to L-dihydroxyphenylalanine(L-DOPA), the rate-limiting step in the synthesis of dopamine⁴⁶. Since TH is a rate-limiting enzyme for the biosynthesis of dopamine, TH activity is progressively decreased following the loss of dopamine neurons in the substantia nigra in the patients with

Parkinson disease⁴⁷. TH immunohistochemistry has widely been used as an important method of detecting the injury or death of dopaminergic fibers and cell bodies^{48,49}.

Previous studies using rats showed that acupuncture at the GB₃₄, LR₃ and ST₃₆ acupoints reduced the degeneration of dopaminergic neurons induced by 6-hydroxydopamine(6-OHDA)⁵⁰. GB₃₄ and LR₃ also protected against MPTP induced dopaminergic neuronal cell damage⁴¹. For the present study, we chose the *Sinsu*(BL₂₃) acupoint because PD is a brain disease, and in Oriental medicine the brain is the sea of marrow nourished by Kidney essence. BL₂₃ is the Kidney transport point that is the specific point on the back where the qi of the Kidney is infused⁵¹. Acupuncture at BL₂₃ lessens the memory loss and decreased immune responses that accompany aging. BL₂₃ can also increase the release of 5-HT⁵². However, no study has examined the effect of BL₂₃ on Parkinson disease to date.

In our study, BVA inhibited MPTP-induced loss of TH-IR neurons and activation of MAC-1-IR neurons in the SNpc, in a dose dependent manner. These results demonstrate that BVA possess a potent suppressive effect on microglial activation and suggest that BVA may offer substantial therapeutic potential for the treatment of neurodegenerative diseases that are accompanied by microglial activation.

HSP70-IR cells were not observed in the SNpc of any of the tested groups. However, since the only observation was made 7days after the last MPTP injection, further research, including observation at different time points, is required.

V. Conclusions

In conclusion, we found that BVA at BL₂₃ inhibited MPTP-induced neuronal loss of TH-IR neurons and microglial activation in the SNpc of a mouse PD model, and that this suppression occurred

in a dose dependent manner. However, HSP70-IR neurons were not observed in the SNpc 7days after the fourth MPTP injection.

VI. References

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