

Original Article

Neuroprotective and Anti-inflammatory Effects of Bee Venom Acupuncture on MPTP-induced Mouse

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국문초록

MPTP 유발 파킨슨병 동물 모델에 대한 봉독약침의 신경보호 효과 및 항염증 효과

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목적 : 이 연구는 MPTP 유발 파킨슨병 동물 모델에서 봉독약침의 신경보호 효과 및 항염증 효과를 확인하기 위해 시행되었다.

방법 : C57BL/6 mice에 신경독소인 *1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*(MPTP)를 하루에 2시간 간격으로 MPTP-HCl(20mg/kg per dose)을 4번 복강 내 주입하여 중뇌 흑질의 도파민 신경세포를 파괴한 파킨슨병 동물 모델을 유발하였다. 실험군은 MPTP군, MPTP 현종 BVA군, MPTP 곡지 BVA군, MPTP 신수 BVA군의 4군으로 하였다. 마지막 MPTP 투여 2시간 후에 1차로 봉독약침을 시술하고, 그 후 48시간 간격으로 총 5차 연속 시술하였다. 봉독약침액의 농도는 0.2mg/Kg으로 하였고, 경혈은 양측 현종(GB₃₉), 곡지(LL₁₁), 신수(BL₂₃)를 사용했고, 주입량은 각 경혈당 양측으로 각 20 μ l씩 주입하였다.

항염증작용을 알아보기 위해 TH, MAC-1, iNOS HSP70을, 세포사멸에 대한 신경세포의 보호효과를 알아보기 위해 caspase-3을 면역조직화학법을 사용하여 실시하였다.

결과 : 실험 결과 MPTP 유발 파킨슨병 동물 모델에서 현종·곡지·신수혈에 대한 봉독약침은 TH-Immunoreactivity neuron의 감소와 microglial activation을 억제하였다. 봉독약침군 모두 효과를 보였으나 그 중 현종과 신수혈에서 특히 억제작용이 컸다. MAC-1에서는 현종혈이 억제작용이 컸다. HSP70-IR neuron

· Acceptance : 2010. 5. 19. · Adjustment : 2010. 5. 27. · Adoption : 2010. 5. 28.

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은 곡지에서 유의한 억제작용을 보였으나, iNOS neuron은 모든 군에서 유의한 차이를 보이지 않았다.

또한 세포사멸억제여부 실험에서 봉독약침은 모두 억제작용을 보였으나 특히 곡지자침군에서 caspase-3 발현을 유의하게 억제하였다.

결론 : 이러한 결과는 봉독약침이 MPTP 투여로 인한 중뇌 흑질의 염증에 의한 도파민 신경세포 손상을, 염증을 억제함으로써 항염 효과를 나타냄을 알 수 있으며, 신경세포를 보호하는 활성이 있음을 보여준과 동시에 세포사멸을 억제하는 활성이 있다고 사료된다.

핵심 단어 : Bee venom Acupuncture, Parkinson disease, MPTP(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), macrophageantigencomplex-1, inducible nitricoxidesynthase, apoptosis, Heatshock-protein

I. Introduction

Bee venom acupuncture(BVA) has been traditionally used in Oriental Medicine to relieve pain¹⁾ and to treat chronic inflammatory diseases such as arthritis, rheumatic diseases, immune disorders²⁾, and neurodegenerative diseases such as multiple sclerosis³⁻⁶⁾.

BVA exhibits analgesic, anti-arthritic, and anti-inflammatory effects that are attributable to not only bioactive bee venom(BV) compounds including peptides(melittin, apamin, and adolapin), enzymes (phospholipase A2)⁷⁻⁹⁾, biologically active amines (histamine, epinephrine), several other nonpeptide components including lipids, carbohydrates and free amino acids¹⁰⁾, but also the mechanical effect of acupuncture stimulation.

Recent studies have suggested that BV has anti-inflammatory properties that inhibit the production of inflammatory cytokines and NO in neurodegenerative diseases^{11,12)}.

Several clinical trials and anecdotal reports have recently suggested that BVA may be an effective treatment for demyelinating diseases of the central nervous system(CNS) such as Parkinson's disease (PD) and multiple sclerosis³⁾, although the underlying mechanism of this action remains unknown.

PD is one of the most common movement disorders. The primary neuropathological basis of PD is a severe deficiency of dopamine in the striatum,

resulting from specific loss of nigrostriatal dopaminergic(DA) neurons, the cell bodies of which reside in the substantia nigra pars compacta(SNpc) and the nerve terminals of which project to the striatum¹³⁾. While different mechanisms, including environmental toxins and genetic factors, initiate DA neuronal damage in the SNpc and striatum in PD, there is unequivocal evidence that activation of neuroinflammatory cells¹⁴⁻¹⁷⁾ leads to apoptosis cell death¹⁸⁾, which aggravates the neuro-degenerative process.

Brain inflammation is the common final pathway in PD¹⁹⁾. Central to this inflammation is the activation of microglia, which act as intrinsic immune effectors when the brain is injured^{20,21)}. Microglia are known to be activated in the PD-affected brain, and MPTP(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) - induced microglial activation is associated with oxidative stress²²⁾.

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 MPTP. These mice display a strong microglial response^{23,24)}, accumulation of cytokines, and elevation of reactive oxygen species(ROS)²⁵⁾, which peak prior to the death of DA neurons, suggesting a pivotal role for the microglial response in the cascade of deleterious events that ultimately leads to DA neuronal death in the SNpc of the MPTP mouse model with PD²⁶⁾.

The heat shock proteins(HSPs) represent an

important cellular protective mechanism against a variety of stresses and insults²⁷⁻²⁹. Adaptive protection was assessed by the expression of heat shock protein(HSP) 70-IR neurons.

We examined 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 synthesis and (b) the effect of BVA is acupoint dependent and (c) the apoptosis is associated with the mechanism of BVA. Microglial activation and neuroinflammation were measured by the expression of macrophage antigen complex (MAC)-1 and inducible nitric oxide synthase(iNOS)-IR neurons. Apoptosis was measured by the expression of caspase 3-IR neurons in relation to MPTP-induced DA neuronal loss in the SNpc.

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. Before experiments, the mice were acclimated for 2 weeks in cages at 21°C and were provided with water and food ad libitum. 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, Kyung Hee University. At the beginning of the experiment, the animals were randomly divided into four groups: MPTP group, MPTP GB₃₉ BVA group, MPTP LI₁₁ BVA group and MPTP BL₂₃ BVA group. All mice (six per group) received an intraperitoneal(i.p.) injection of $\times 4$ (Sigma, St. Louis, MO, USA) in saline every 2hr, over an 8hr period, in 1day³⁰. MPTP was dissolved in 5 μ l saline and an i.p. injection was performed as previously described³⁰ using a 30- μ l Hamilton syringe with a 30-gauge needle. The animals were sacrificed at one day after the last BVA(Fig. 1).

2. Treatment with diluted bee venom

BV was diluted to doses of 0.2mg/kg in 40 μ l of normal saline; 20 μ l of each dose were subcutaneously administered bilaterally into each GB₃₉, LI₁₁ and BL₂₃.

GB₃₉, LI₁₁ and BL₂₃ began 2 hr after the last MPTP i.p. injection and then resumed at 48 hr intervals for a total of 5 times until the mice were sacrificed, 10 days after the last MPTP injection. For this procedure, the mice in the MPTP+BVA groups were immobilized, and BV was administered into GB₃₉, LI₁₁ and BL₂₃.

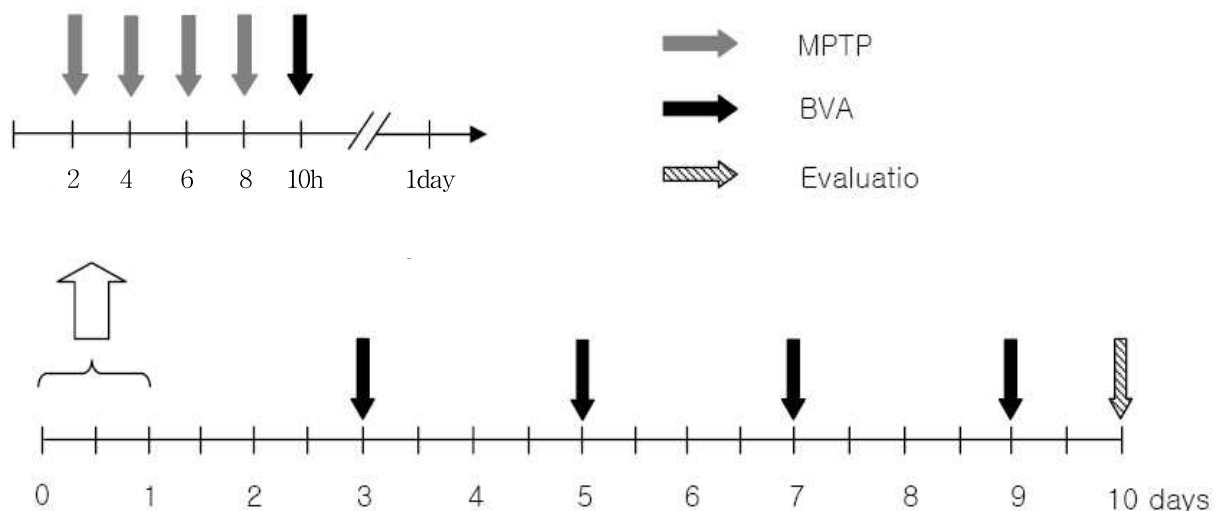


Fig. 1. MPTP injection and BVA schedule

3. Tissue preparation and immunohistochemistry

The mice in all groups were sacrificed by anesthesia with pentobarbital sodium(60mg/kg, i.p.) 10 days after the last MPTP injection and perfused transcardially with paraformaldehyde(4% in 0.1 M 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 30 μ m slices for histological analysis.

For the immunohistochemistry, brain sections were incubated with one of the following antibodies: (1) rabbit anti-TH antibody(Chemicon; 1 : 4,000), (2) rabbit anti-MAC-1 antibody(Serotec; 1 : 500), (3) rabbit anti-heat shock protein(HSP70) antibody(Chemicon; 1 : 2,000), (4) mouse anti-iNOS (Upstate; 1 : 2,000) and (5) rabbit anti-caspase 3 antibody(Cell Signaling Technology; 1 : 1,000). Brain sections were treated with primary antibody at room temperature for 16hr, 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 three TH-immunostained mesencephalic sections at the widest dimension of the SNpc at AP-3.16(Franklin and Paxinos, 1996), 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, iNOS, HSP70, and caspase 3-IR neurons

in 10 sections from the SNpc.

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 curve³¹. During full measurement of the optical density, the optical voltage was maintained at a constant level.

5. Statistical analysis

Means and S.D. were calculated for the estimated numbers of TH-IR neurons and the optical density of MAC-1-IR, iNOS-IR, HSP70-IR, and caspase-3-IR positive neurons. To rule out a possible change in SNpc volume as an influencing factor, the numbers of TH-IR neuron is expressed as a ratio of 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 CPU

Immunohistochemical staining with TH antibody was performed on the brain samples collected from each group 10 days 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 MPTP+BVA groups (Fig. 2A).

The survival TH-IR neurons in the CPU of the MPTP group were 77~83% compared with the

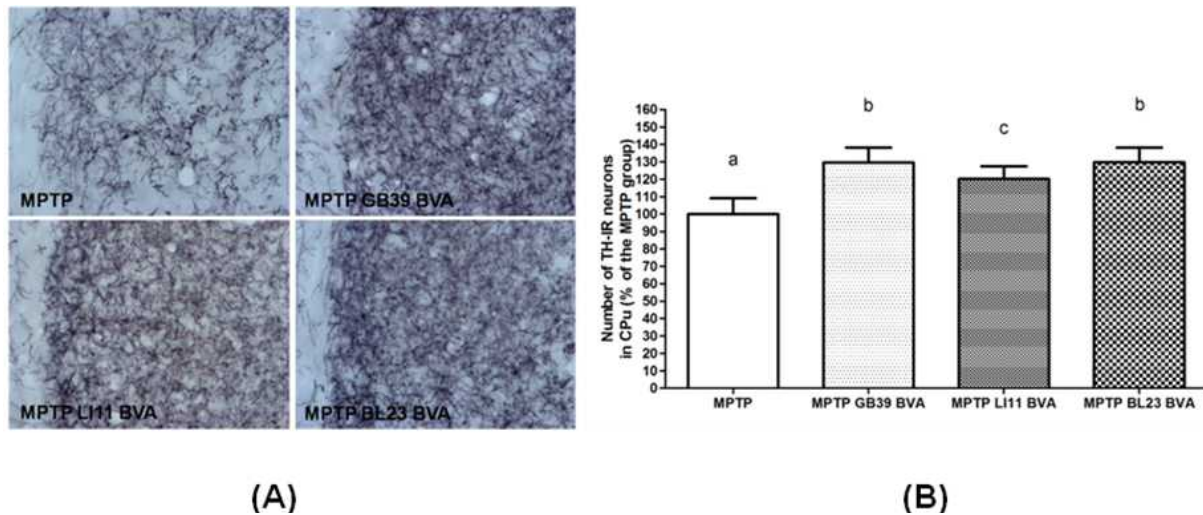


Fig. 2. Effects of BVA on MPTP-induced neuronal loss of tyrosine hydroxylase(TH)-immunoreactive(IR) neurons in the caudate-putamen(CPu)

(A) TH-IR neurons on day 10 after the 4thMPTP injection.
 (B) Levels of the number of TH-IR neurons on day 10.

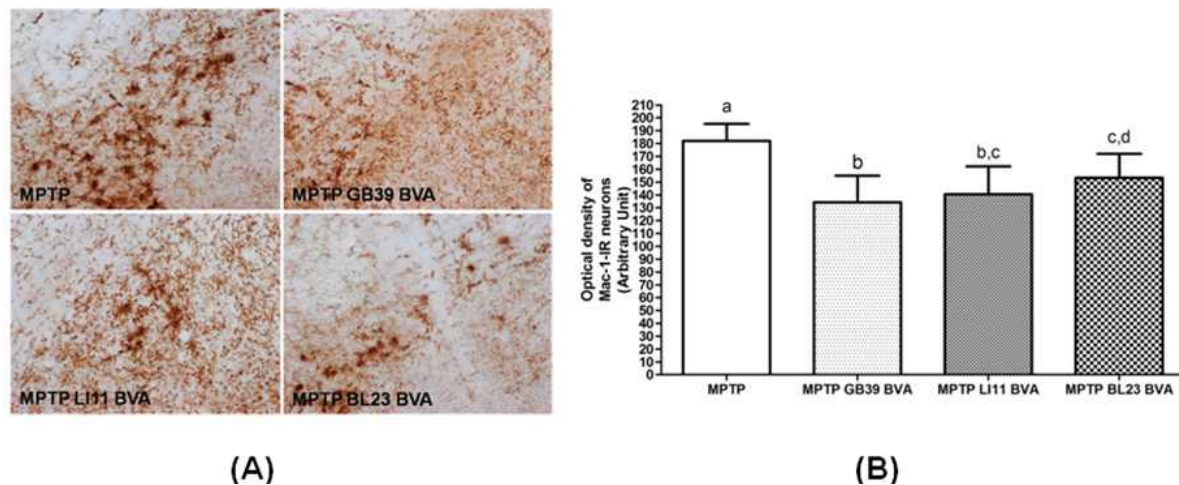


Fig. 3. Effects of BVA on MPTP-induced microglial activation of MAC-1-immunoreactive(IR) neurons in the substantia nigra pars compacta(SNpc)

(A) Mac-1-IR neurons on day 10 after the 4thMPTP injection.
 (B) The optical density levels of MAC-1-IR neurons on day 10.

other groups. In the MPTP GB₃₉ and BL₂₃ BVA group, the survival TH-IR neurons was 30% greater than in the MPTP group, which was statistically significant(Fig. 2B).

The levels are expressed as the average number of TH-IR caudate-putamen neurons per section in the MPTP group. The average number was significantly greater in all MPTP+BVA groups than in the MPTP group. Values are means±S.D.(Bonferroni's multiple range test, $\alpha = 0.05$).

2. BVA inhibits the MPTP-induced activation of MAC-1-IR neurons in the SNpc

The SNpc is relatively rich in microglia compared with other brain regions^{32,33}. Previous studies have suggested that activation of microglia may trigger or participate in the neurodegenerative processes in PD¹⁹. To determine whether the beneficial effect of BVA was associated with inhibition of the

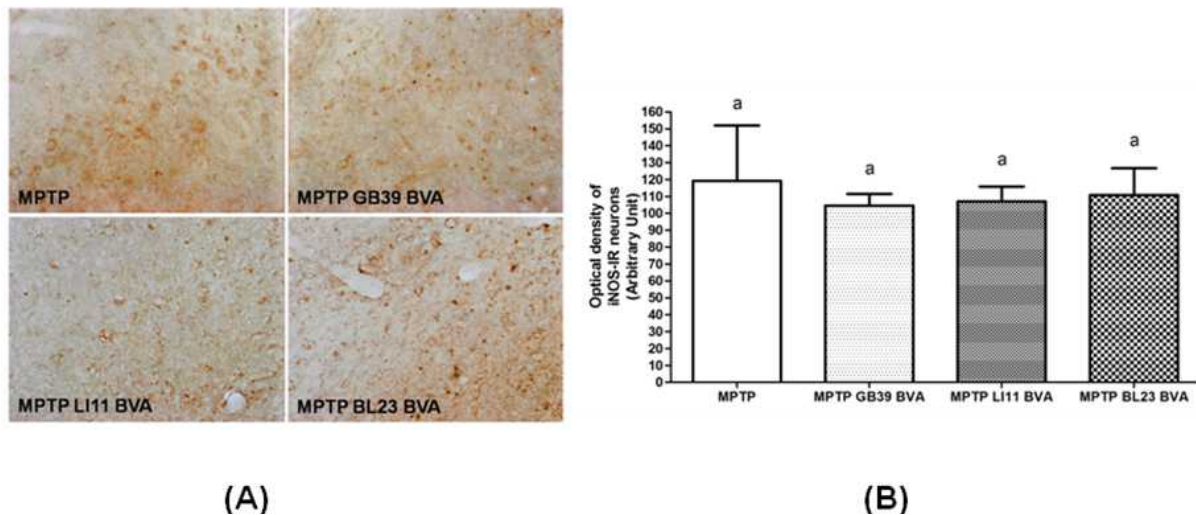


Fig. 4. Effects of BVA on MPTP-induced microglial activation of iNOS-immunoreactive(IR) neurons in the substantia nigra pars compacta(SNpc)

(A) INOS-IR neurons of on day 10 after the 4thMPTP injection.
 (B) The optical density levels of iNOS-IR neurons on day 10.

MPTP-induced glial response, we examined the expression of MAC-1, a marker of microglial activation, 10 days after the last MPTP injection. In the MPTP 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 decreased in the MPTP+BVA groups, compared with the MPTP group(Fig. 3A).

The activation of MAC-1-IR neurons was decreased in the MPTP BVA group compared with the MPTP group. In particular, the activation of MAC-1-IR neurons was significantly decreased in the MPTP GB₃₉ BVA group compared with the MPTP group(Fig. 3B)

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. The optical density was statistically significantly lower in all MPTP+BVA groups than in the MPTP group. Data are expressed as mean±S.D. of the average optical density for each section. The means with same letter over the bars is not significantly different(Bonferroni's multiple range test, $\alpha = 0.05$).

3. Effects of BVA on MPTP-induced microglial activation of iNOS-IR neurons in the substantia nigra pars compacta(SNpc)

Because iNOS-derived nitric oxide plays a major role in inflammation-mediated neurodegeneration, we measured the expression of iNOS in the SNpc. To date, one of the best characterized cytotoxic mechanisms induced by proinflammatory cytokines in PD is the activation of iNOS, which mediates the synthesis of high levels of nitric oxide shown to be toxic to neurons^{4,7,20}. MPTP i.p. injection to mice produced a robust gliosis in the SNpc associated with significant up-regulation of iNOS, and these changes paralleled MPTP-induced DA neuroinflammation^{10,31}.

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 iNOS-IR neurons per section(Fig. 4A). The expression and the optical density did not show any significant differences among all groups.

Data are expressed as mean±S.D. of the average optical density for each section. The means with

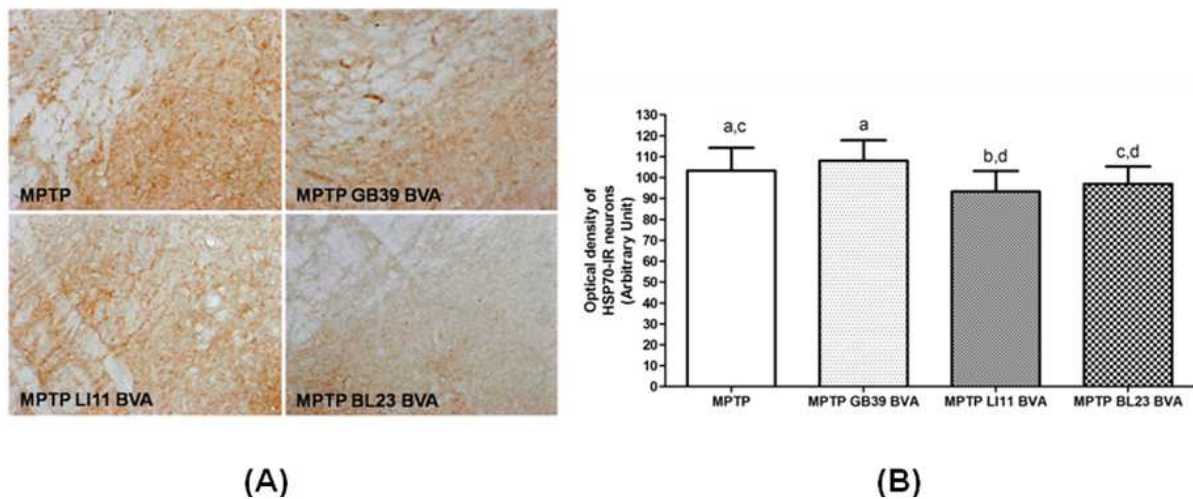


Fig. 5. Effects of BVA on MPTP-induced microglial activation of HSP70-immunoreactive(IR) neurons in the substantia nigra pars compacta(SNpc)

(A) HSP70-IR neurons on day 10 after the 4thMPTPi.p.injection.

(B) The optical density levels of HSP70-IR neurons on day 10

same letter over the bars is not significantly different(Bonferroni's multiple range test, $\alpha = 0.05$).

4. Effects of BVA on MPTP-induced microglial activation of HSP70-immunoreactive(IR) neurons in the substantia nigra pars compacta(SNpc)

Activation of heat shock protein(HSP) synthesis in neurons is an important mechanism for adaptive protection of cells in MPTP-induced neurotoxicity^{29,34}. To define the temporal relationship between the effect of BVA and HSP synthesis, HSP70 was assessed in MPTP-induced DA cell loss on day 10 day after the last MPTP injection. The optical density was decreased in the MPTP LI₁₁ BVA group than in the MPTP group(Fig. 5).

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 HSP70-IR neurons per section. The optical density was lower in the MPTP LI₁₁ BVA group than in the MPTP group. Data are expressed as mean \pm S.D. of the average optical density for each section. The means with same letter over the bars is not significantly different (Bonferroni's multiple range test, $\alpha = 0.05$).

5. Effects of BVA on the expression of caspase 3 - IR neurons in MPTP-induced SNpc neurodegeneration

Apoptosis represents a morphologically and biochemically distinct form of programmed cell death that was originally recognized to play a considerable role in developmental cell death¹⁸. Apoptotic cell death was found to be initiated within 72h of the first injection of the neurotoxin, and to peak 24 h after the last MPTP injection³⁵. Caspase 3-IR neurons are effectors of SNpc neuron apoptosis³⁶, and caspase 3-IR neuron expression is more sensitive to the pathological process in the SNpc, which is induced by MPTP injection³⁷.

The optical density of caspase 3-IR neurons was significantly decreased in the MPTP LI₁₁ BVA group compared to the MPTP and other MPTP BVA groups(Fig. 6).

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 caspase 3-IR neurons per section. The optical density was lower in all MPTP+BVA groups than in the MPTP group where the density of MPTP LI₁₁ BVA was the lowest. Data are expressed as mean \pm S.D. of the average optical

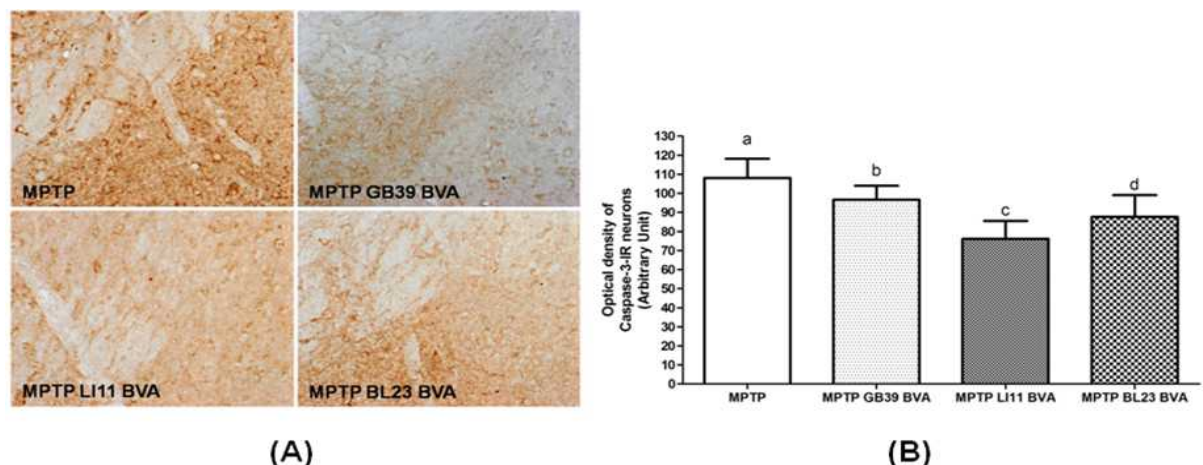


Fig 6. Effects of BVA on MPTP-induced microglial activation of caspase 3-immunoreactive(IR) neurons in the substantia nigra pars compacta(SNpc)

(A) caspase 3-IR neurons on day 10 after the 4thMPTPi.p.injection.
 (B) The optical density levels of caspase 3-IR neurons on day 10.

density for each section. The means with different letter over the bars is significantly different in every pair(Bonferroni's multiple range test, $\alpha = 0.05$) In the Bonferroni's multiple range test, the difference between MPTP group and MPTP LI₁₁ BVA group was the largest(mean difference = 31.920 ± 2.689 , $p=0.000$) among every pairwise comparison.

IV. Discussion

PD is the result of a quite specific and progressive neurodegeneration of pigmented nigrostriatal 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^{38,39}.

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 stress⁴⁰⁻⁴³. Recently, however, there has been increasing recognition of the possible major role of neuroinflammation in the

pathogenesis of PD⁴⁴, induced by exposure to either infectious agents or toxicants with proinflammatory 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 function⁴⁵. They are activated in the event of infection, inflammation, trauma, ischemia and neurodegeneration in the CNS⁴⁶. It has been shown that glial cells are activated in the PD brain and MPTP-induced glial activation in association with oxidative stress²². 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 neurotoxicity⁴⁷. Recent studies suggested that BV has anti-inflammatory properties that inhibit production of inflammatory cytokines and NO in the activated microglia^{11,12}. Acupuncture also inhibits microglial activation and inflammatory events in the MPTP-induced mouse model⁴⁸.

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 temperature⁴⁹. HSPs are a group of highly conserved stress proteins that play an important role in maintaining body self-stability. The main func-

tions 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 stimulation⁵⁰. It is known that a plethora of insults other than heat stress have 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 neurotoxicity³⁴. Moxibustion and electroacupuncture can induce the synthesis of heat shock protein 70^{51,52}. However, to date no studies have examined the relationship between BVA and HSPs.

In the present study, BVA resulted in preservation of TH-IR neurons on day 10 after MPTP *i.p.* injection. A strong relationship was observed between the damage incurred to DA neurons and the intensity of MAC-1 expression that was present in the activated microglia of the SNpc. BVA also decreased the expression of caspase 3-IR neurons, which are responsible for developmental cell death¹¹.

Therefore, along with the cited findings for BVA, our results supported the hypothesis that BVA may attenuate inflammatory activities in the SNpc induced by MPTP administration. Considering the role of microglia in mediating neurodegeneration, these results suggest that BVA might be an attractive alternative treatment to suppress the development or progression of chronic inflammatory diseases of the central nervous system, such as PD, multiple sclerosis, and amyotrophic lateral sclerosis.

This study showed that MPTP-induced mouse model to examine whether BVA inhibits the loss of tyrosine hydroxylase(TH)-positive neurons as a result of its inhibition of microglial activation or induction of HSP70 and the effect of BVA is acupoint dependent. Microglial activation was measured by the expression of MAC-1. Due to the strain-dependent sensitivity to MPTP⁵³, 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 dopamine⁵⁴. 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 patients with PD⁵⁵. TH immunohistochemistry has widely been used as an important method of detecting the injury or death of dopaminergic fibers and cell bodies^{56,57}.

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)⁵⁸. GB₃₄ and LR₃ also protected against MPTP induced dopaminergic neuronal cell damage⁴⁸.

In our study, BVA inhibited MPTP-induced loss of TH-IR neurons and activation of MAC-1-IR neurons in the SNpc. These results demonstrate that BVA possesses 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 lower in the MPTP LL₁₁ BVA group than in the MPTP group, but there are no significant differences between the groups.

V. Conclusions

We found that BVA inhibited MPTP-induced neuronal loss of tyrosine hydroxylase(TH)-positive neurons as a result of its inhibition of microglial activation or induction of HSP70 in the SNpc of a mouse PD model. And the effect of BVA is acupoint dependent.

In addition, BVA suppressed microglial activation, which was associated and colocalized with an increase in MAC-1, iNOS and HSP-70 expression. Furthermore, BVA prevented MPTP-induced apo-

ptosis of DA neurons via caspase-3 inhibition.

VI. References

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