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Original Article

Inhibitory Effect of Bee Venom on Lipopolysaccharide-induced Memorial Impairment and Acetylcholine Esterase, Secretase Activity

Kwon Dae-hyun and Song Ho-sueb

Department of Acupuncture & Moxibustion, College of Oriental Medicine, Kyungwon University

Abstract

Alzheimer's disease (AD) is the most prevalent form of neurodegenerative disease associated with aging in the human population. This disease is characterized by the extracellular deposition of beta-amyloid peptide (Aβ) in cerebral plaques. Aβ is derived from the β-amyloid precursor protein (APP) by the enzymes, β- and γ-secretase. Compounds that β- or γ-secretase inhibit activity, can reduce the production of Aβ peptides, and thus have therapeutic potential in the treatment of AD. Increasing body of evidence has been demonstrated that Bee Venom(BV) Acupuncture could compete with complex protein involving in multiple step of NF-κB activation and exert the anti-inflammatory potential of combined inhibition of the prostanoid and nitric oxide synthesis systems by inhibition of IKK and NF-κB. In this study, I investigated possible effects of BV on memory dysfunction caused by lipopolysaccharide (LPS) and Aβ through inhibition of secretases activities and Aβ aggregation. I examined the improving effect of BV on the LPS (2.5 mg/Kg, i.p.)-induced memory dysfunction using passive avoidance response and water maze tests in the mice. BV (0.84, 1.67 μg/ml) reversed the LPS-induced memorial dysfunction in dose dependent manner. BV also dose-dependently attenuated LPS-induced β and γ-secretase activities in cerebral cortex and hippocampus of the mice brain. This study therefore suggests that BV acupuncture method may be useful for prevention of development or progression of AD.

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· **Corresponding author** : Song Ho-sueb, Director of Acupuncture & Moxibustion Department, Kyungwon University Seoul Hospital
Tel. 82-2-425-3456 E-mail : hssong70@kyungwon.ac.kr

Key words : *Aβ; LPS; Alzheimer's disease; Acetylcholine esterase; secretase; memory*

Ⅰ. Introduction

Alzheimer's disease(AD) is an age-related neurodegenerative disease characterized by progressive degeneration and loss of neurons in the brain[1]. Beta-amyloid peptide (Aβ) can be accumulated as insoluble extracellular deposits in senile plaques, the neuropathological hallmarks of AD[2-3]. This hydrophobic polypeptide is proteolytically produced from an amyloid precursor protein[4]. Several lines of evidence have been shown that Aβ is considered to have a causal role in the development and progress of AD[5-7]. In vitro and in vivo experimental data indicate that Aβ can directly induce neuronal cell death[8-9]. The mechanism underlying Aβ-induced neurotoxicity is complex, but enhanced oxidative stress may be implicated in increment of the vulnerability of neurons causing apoptotic cell death[8,10].

Consideration of the strong association between Aβ and AD, it seems that therapeutic strategies to reduce the levels of Aβ in the brain should be beneficial for the treatment of AD. Inhibition of the enzymes that generate Aβ may be one great interesting strategy. Aβ is a product of catabolism of the large type-I membrane β-amyloid precursor protein (APP) by proteases, called β- and γ -secretase[11-12]. Recently, secretase is an attractive drug target for AD. Compounds that alter the proteolytic cleavage of APP, including those that inhibit β- or γ-secretase activity, can reduce the production of Aβ peptides and may have therapeutic potential in the treatment of AD[13-14].

Reviewing these two articles, the neuroprotective effect of green tea was thought to be related with the inhibition of inflammatory response.

According to a report issued recently, Inhibition of NF- κB activation by BV, through the direct ability of BV and/or melittin to bind to p50, may be an important molecular mechanism in the suppressive effect of BV on inflammatory reactions. The potency of BV (or melittin) in the inhibition of the inflammatory response may be of great benefit in degenerative and inflammatory diseases[15].

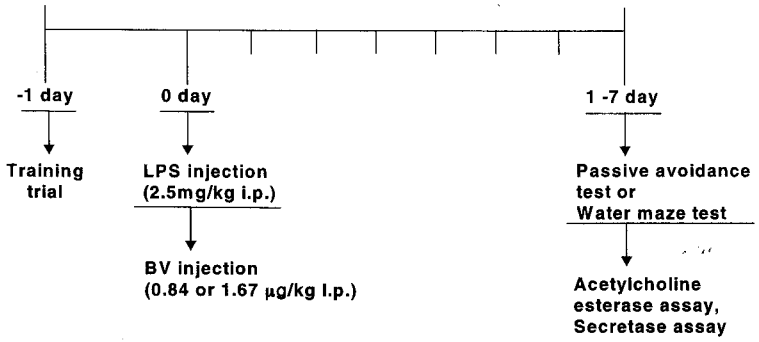
Since previous our ischemia-reperfusion animal model, 1.5 and 2 % BV treatment for 2 weeks showed neuroprotective effect[16-17], BV are also expected to play a major role in exerting the neuroprotective effect via inhibition of inflammatory reaction in the NF-kB signal pathway and the effect of BV on the secretase activity and its relevance with memory function in vivo has been not reported yet. Therefore, in the present study, I investigated whether of BV acupuncture could improve recognition ability and inhibit secretase activity in mice models.

Ⅱ. Materials and Methods

1. Bee Venom(BV)

BV was employed from *You-Miel Nongwon* (Hwasoon, South Korea). The composition of the BV was follows: 45-50% melittin, 2.5-3% apamin, 2-3%. MCD peptide,

12% PLA2, 1% lyso-PLA2, 1-1.5% histidine, 4-5% 6pp lipids, 0.5% secarpin, 0.1% tertiapin, 0.1% procamine, 1.5-2% hyaluronidase, 2-3% amine, 4-5% carbohydrate, and 19-27% other, including protease inhibitor, glucosidase, invertase, acid phosphomonoesterase, dopamine, norepinephrine, and unknown amino acids, with>99.5% purity.



Scheme 1. Time course of the treatment, training and tests

2. LPS

LPS (Sigma, St. Lous, MO. USA) were dissolved and aliquots were stored at -20℃until use.

3. Animals

Male mice IcrTacSam:ICR (Samtako, Gyeonggi-do, Korea) were maintained in accordance with the National Institute of Toxicological Research of the Korea Food and Drug Administration guideline for the care and use of laboratory animals. All mice were housed in a room that was automatically maintained at 21~25℃ and relative humidity (45~65%) with a controlled light-dark cycle.

4. Memory impairment mice model

I decided to use the method of administrating LPS alone into intraperitoneum(i.p.), reviewing articles related with memorial impairment[18-25].

I therefore used the latter method as an AD mice model. The LPS (final concentration of 2.5 mg/Kg) were dissolved and diluted in saline. LPS solution were performed according to the procedure established by Laursen and Belknap[26]. Briefly, each mouse was injected (without anesthesia) at i.p..

5. Experimental design

The inducers (2.5 mg/Kg of LPS) or vehicle (saline) were administrated i.p. Mice(18g) were educated on three times of training trials and then the inducers(2.5 mg/Kg of LPS) were injected at i.p. On the experimental groups, BV Acupuncture (0.84 or 1.67 μg/kg) was done at i.p. as well. Again after memory impairment induced and the behavioral tests were 1 day after injection. Learning and memory capacity was assessed using two separate tests (passive avoidance and water maze tests), and their mechanism study was judged as biochemical test (β- and γ- secretase activities) in separated brain regions (cerebral cortex and hippocampus) of the sacrificed mice. The experimental schedule was shown in Scheme 1. The time schedule of treatment and assay.

6. Behavioral tests

1) Passive avoidance performance test

The passive avoidance test is widely accepted as a simple and rapid method for memory test. The active avoidance response was determined using a "step through" apparatus that is consisted of an illuminated and a dark compartment (each 4 x 13 x 10 cm) adjoining each other through a small gate with a grid floor, 2.5 mm stainless steel rod set 7 mm apart. On a training trial, the ICR mice were placed in the illuminated compartment facing away from the dark compartment. When the mice moved completely into the dark compartment, it received an electric

shock (0.4 mA, 2 s duration). Then the mice were returned to their home case. At 24 hr later, mice were given LPS (2.5 mg/Kg, i.p.). After 1 day treatment of LPS, the mice were placed in the illuminated compartment and the latency period to enter the dark compartment defined as "retention" was measured. The time when the mice entered in the dark compartment were recorded and described as transfer latency (TL). The retention trials were set at a limit of 600 s. of cut-off time.

2) Water maze test

The water maze test is also widely accepted method for memory test, we was performed this test as described by previously[27]. Maze testing was performed by the SMART-CS (Panlab, Barcelona, Spain) program and equipment. A circular plastic pool (height: 35 cm, diameter: 100 cm) was filled with milky water kept at 22~25℃. An escape platform (height: 14.5 cm, diameter: 4.5 cm) was submerged 0.5~1 cm below the surface of the water in position. On training trials, the mice were placed in a pool of water and allowed to remain on the platform for 10 s and were then returned to the home cage during the second-trial interval. The mice that did not find the platform within 120 s were placed on the platform for 10 s at the end of trial. At 24 hr later, mice were given LPS (2.5 mg/Kg, i.p.). 1 day after treatment of LPS, the mice were placed in the water pool which platform was taken out. They were allowed to swim until they sought escape platform. Escape latency, escape distance, swimming speed and swimming pattern of each mouse were monitored by a camera above the center of the pool connected to a SMART-LD program (Panlab, Barcelona, Spain).

7. Biochemical tests

1) Brain collection and preservation

After behavior test, animals were perfused with PBS under inhaled chloroform anesthetization. The brains were immediately collected in the same manner and frozen stored at -20℃, and cut and separated into cortical and hippocampal regions. All the brain regions were immediately stored at -80℃, and used to measure activities of acetylcholine esterase, β- and γ-secretase assay.

2) Acetylcholine esterase assay

The assay was modified based on Ellman method(Fig. 3)[28]. Briefly, 5-10 ml of sample was mix with 200 ml of reaction buffer (50mM Tris-HCl pH 8.0 containing 0.01 % DTNB, 0.02% acetylthiocholine, 0.1mM iso-OMPA). The activity of the enzyme was determined after 10 min of incubation at 37℃. The optical density was measured at 405 nm corresponding to the quantity (nmole) of the acetylthiocholine, which was hydrolyzed to thiocholine for 1 min per 1ml enzyme solution. Enzyme activity is expressed in unit of nmole/min/mg protein.

3) β- and γ-secretase assay

The total activities of β- and γ-secretase present in cortical and hippocampal reigns which were determined using commercially available β-secretase fluorescence resonance energy transfer (BACE 1 FRET) assay kit (PANVERA, Madison, USA) and γ-secretase activity Kit (R&D systems, Wiesbaden, Germany) according to the manufacture's protocols, respectively. The cerebral cortex and hippocampus were homogenized in cold 1× cell extraction buffer (ready for use in the kit) to yield a final protein concentration of 1 mg/ml.

To determine β-secretase, 10 μl lysate was mixed with 10 μl BACE1 substrate (Rh-EVNLDAEFK-Quencher), and then the reaction mixture was incubated for 1 hr at room temperature in the 96 well flat bottom microtitre plate. The reaction was stopped by addition of 10 μl BACE1 stop buffer (2.5 M sodium acetate). The formation of fluorescence was read with Fluostar galaxy fluorometer (excitation at 545 nm and emission at 590 nm) with Felix software (BMG Labetchnologies,

Offenburg, Germany). The enzyme activity was linearly related to the increase in fluorescence. The enzyme activity was expressed as nM produced substrate which was determined by the formation of fluorescence per mg protein per min.

To determine γ-secretase, 50 μl lystate was mixed with 50 μl reaction buffer. The reaction mixture was then incubated for 1 hr in the dark at 37℃. 5 μl substrate was added after the periods of incubation time to stop the reaction. Cleaved substrate by γ-secretase was conjugated to the reporter molecules EDANS and DABCYL, and released fluorescent signal. This formation of fluorescence was read with Fluostar galaxy fluorometer (excitation at 355 nm and emission at 510 nm) with Felix software (BMG Labetchnologies, Offenburg, Germany). The level of γ-secretase enzymatic activity is proportional to the fluorometric reaction, and the γ-secretase activity was expressed the produced fluoresce unit. All controls, blanks and samples were run in triplicate.

8. Statistics

Data were analyzed using one-way analysis of variance followed by Tukey's test as a post hoc test(* ; $p<0.05$.).

Ⅲ. Results

1. Effect of BV on the LPS-induced memory impairment by passive avoidance performance test

Since previous our ischemia-reperfusion animal model, 1.5 and 2 % BV treatment for 2 weeks showed neuroprotective effect[16-17].

LPS (2.5 mg/Kg, i.p.) significantly decreased the step through TL in comparison to that of control mice. Memory impairment by treatment of LPS was significantly recovered in BV-treated mice determined in 1, 3 and 7 day treatment. Control (vehicle) group exhibited TL about 463.30 ± 59.46, 525.90 ± 40.73 and 502.00 ± 52.72 s (*n=10*), whereas LPS-treated group decreased to 238.10 ± 73.77, 238.10 ± 83.74 and 351.10 ± 72.98 s (*n=10*), respectively. The LPS decreased TL in the mice injected with 0.84 μg/Kg BV were recovered to 340.80 ± 70.68, 340.80 ± 75.92 and 495.60 ± 46.17 s (*n=10*) in dose dependent manner. The LPS decreased TL in the mice injected with 1.67 μg/Kg BV were also recovered to 374.30 ± 72.20, 374.30 ± 56.70 and 454.60 ± 73.28. (Fig. 1, 2, 3)

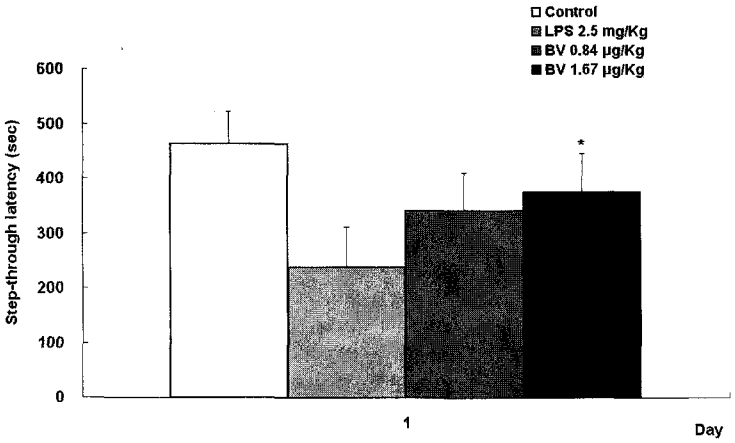


Fig. 1. The effect of BV on memorial impairment induced by LPS in the step-through type passive avoidance performance test. The mice were performed on the step-through transfer latency to the dark compartment at 1 day after training trial as mentioned in materials and methods. LPS (2.5 mg/kg i.p.) was treated 24 hr before assay as a positive control. Each value means ± S.E. from three separated experiments (n=30). * Significantly different from positive LPS treated control ($p<0.05$)

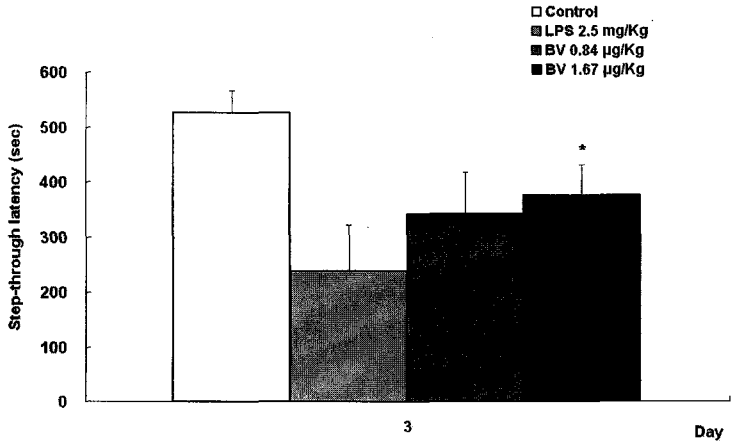


Fig. 2. The effect of BV on memorial impairment induced by LPS in the step-through type passive avoidance performance test. The mice were performed on the step-through transfer latency to the dark compartment at 3 day after training trial as mentioned in materials and methods. LPS (2.5 mg/kg i.p.) was treated 24 hr before assay as a positive control. Each value means ± S.E. from three separated experiments (n=30). * Significantly different from positive LPS treated control ($p<0.05$)

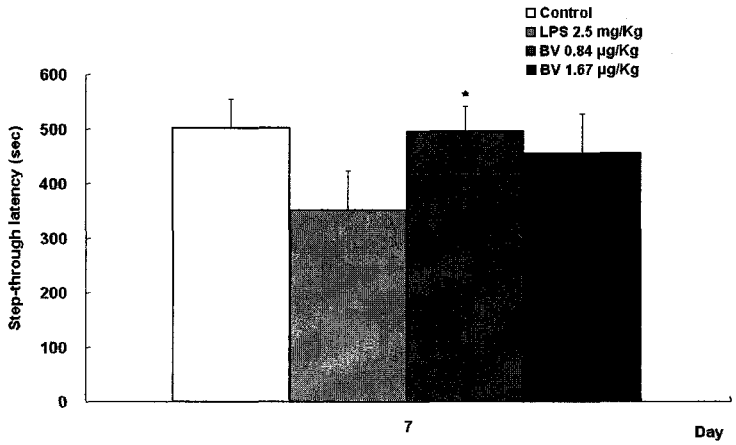


Fig. 3. The effect of BV on memorial impairment induced by LPS in the step-through type passive avoidance performance test. The mice were performed on the step-through transfer latency to the dark compartment at 7 day after training trial as mentioned in materials and methods. LPS (2.5 mg/kg i.p.) was treated 24 hr before assay as a positive control. Each value means ± S.E. from three separated experiments (n=30). * Significantly different from positive LPS treated control ($p<0.05$)

2. Effect of BV on LPS-induced memory impairment by Morris water maze test

To further examine the cognitive-enhancing activity of BV, we determined whether BV can improve spatial memory function using Morris water maze. The mice were trained by water maze test. 1 day after training, LPS (2.5 mg/Kg, i.p.) were then injected into mice. LPS-treated mice slowly arrived at the location of the platform compared to control, but 0.84 and 1.67 μg/Kg BV-treated groups significantly inhibited the effects of LPS on escape distance and latency determined 1, 3 and 7 days after injection of LPS for memorial impairment induced. LPS-induced mice exhibited latency to the platform about 71.20 ± 31.34 s, 53.37 ± 17.83 s and 48.57 ± 25.62 s (*n=10*), respectively. However, those values in the

mice injected with 0.84 μg/Kg BV were decreased to50.65 ± 15.17 s, 43.51 ± 22.15 s and 32.42 ± 11.81 s (*n=10*) in dose dependent manner at 1, 3 and 7 days trial after treatment of LPS and on the mice injected with 1.67 μg/Kg BV were significantly decreased to 40.10 ± 11.40 s, 27.93 ± 5.67 s and 26.90 ± 7.43 s (*n=10*) in close dependent manner at 1, 3 and 7 days trial after memorial impairment induced.(Fig. 4, 5, 6)

3. Effect of BV on the activities of acetylcholine esterase activities

To elucidate the action mechanism of BV on cognitive-enhancing effect, I determined acetylcholine esterase activity in cerebral cortex and hippocampus of the mice. The activity of the acetylcholine esterase in the hippocampus of LPS-treated mice incresed(about 50% over the level of control), whereas that level in the cerebral cortex were no more than 17% of increase compared to the control.

Fig. 4. The effect of BV on memorial impairment induced by LPS in the water maze test. The mice were given the swimming test task training trial. Escape latencies (s) of test in locating the invisible platform was performed at 1 day after training. LPS (2.5 mg/kg, i.p.) was treated 24 hr before test as a positive control. Each value means ± S.E. from three separated experiment (n=30)
* Significantly different from positive LPS treated control ($p<0.05$).

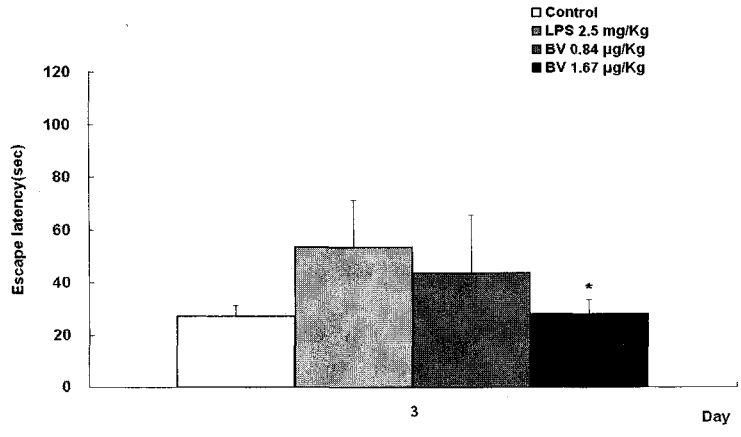


Fig. 5. The effect of BV on memorial impairment induced by LPS in the water maze test. The mice were given the swimming test task training trial. Escape latencies (s) of test in locating the invisible platform was performed at 3 days after training. LPS (2.5 mg/kg, i.p.) was treated 24 hr before test as a positive control. Each value means ± S.E. from three separated experiments (n=30)
* Significantly different from positive LPS treated control ($p<0.05$).

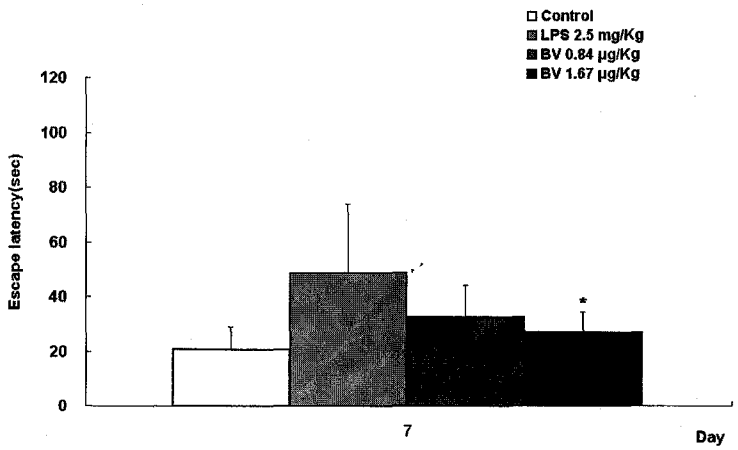


Fig. 6. The effect of BV on memorial impairment induced by LPS in the water maze test. The mice were given the swimming test task training trial. Escape latencies (s) of test in locating the invisible platform was performed at 7 days after training. LPS (2.5 mg/kg, i.p.) was treated 24 hr before test as a positive control. Each value means ± S.E. from three separated experiments (n=30)
* Significantly different from positive LPS treated control ($p<0.05$).

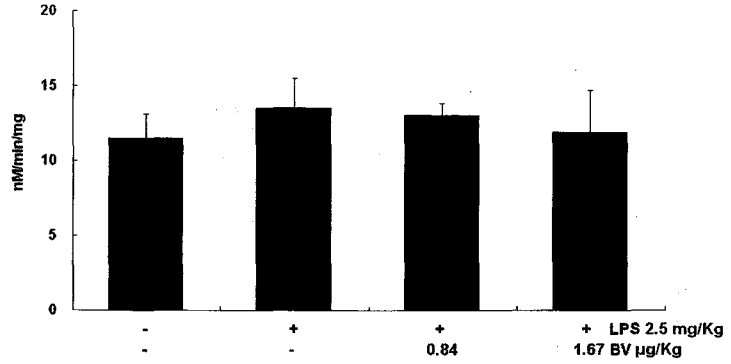


Fig. 7. The effect of BV on acetylcholine esterase activity induced by LPS in cerebral cortex. BV were administrated by i.p., and then the mice were treated LPS (i.p.). The acetylcholine esterase activity was measured in each regions of brain at 7 day after treatment of LPS. acetylcholine esterase activity was presented by the unit of fluorescence which is proportional to enzyme activity. The effect of BV was tested in three independent experiments (n=30)

BV treatment significantly inhibited LPS increased acetylcholine esterase activity in a dose dependent manner in the hippocampus, in 1.67 μg/Kg BV acetylcholine esterase activity was significantly decreased(Fig. 7, 8).

4. Effect of BV on the activities of β- and γ-secretase

To elucidate the action mechanism of BV on cognitive-enhancing effect, I determined secretase activity in cerebral cortex and hippocampus of the mice. The activity of the β- and γ-secretase in the cerebral cortex and hippocampus of and LPS-treated mice generally increased (120 - 230% over the level of control). BV treatment inhibited LPS increased β-secretase in a dose dependent pattern in both cerebral cortex and hippocampus. BV treatment also inhibited LPS increased γ-secretase in a dose dependent pattern in both cerebral cortex and hippocampus. The increased activities were down to control level by higher dose of BV in each secretase of both brain regions.(Fig.9, 10 and Fig. 11, 12)

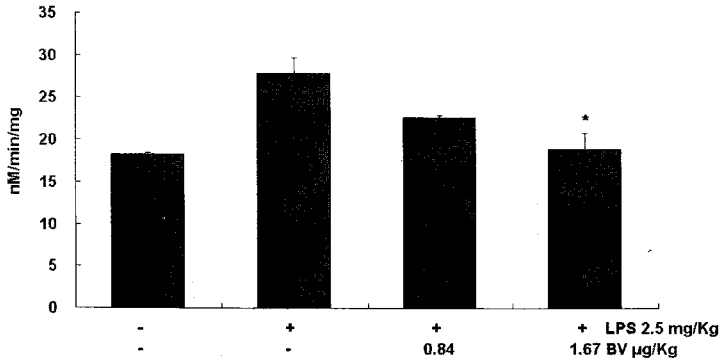


Fig. 8. The effect of BV on acetylcholine esterase activity induced by LPS in hippocampus. BV were administrated by i.p., and then the mice were treated LPS (i.p.). The acetylcholine esterase activity was measured in each regions of brain at 7 day after treatment of LPS. acetylcholine esterase activity was presented by the unit of fluorescence which is proportional to enzyme activity. The effect of BV was tested in three independent experiments (n=30)

* Significantly different from positive LPS treated control ($p<0.05$)

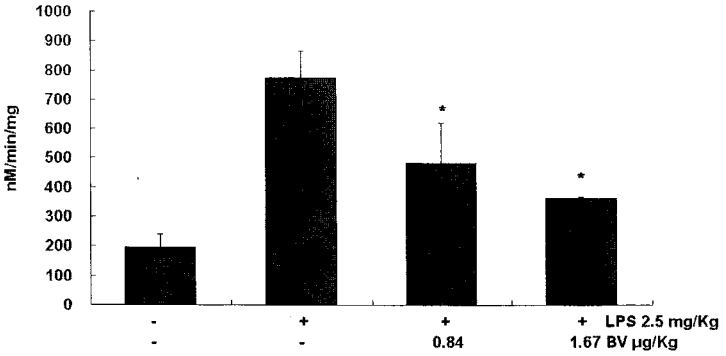


Fig. 9. The effect of BV on β-secretase activity induced by LPS in cerebral cortex. BV were administrated by i.p., and then the mice were treated LPS (i.p.). The β-secretase activity was measured in each regions of brain at 7 day after treatment of LPS. β-secretase activity was presented by the unit of fluorescence which is proportional to enzyme activity. The effect of BV was tested in three independent experiments (n=30)

* Significantly different from positive LPS treated control ($p<0.05$).

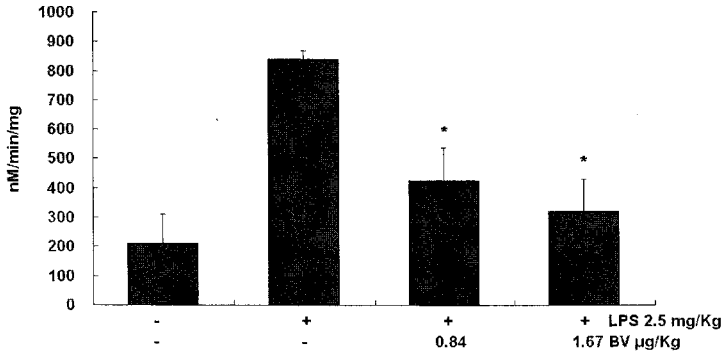


Fig. 10. The effect of BV on β-secretase activity induced by LPS in hippocampus. BV were administrated by i.p., and then the mice were treated LPS (i.p.). The β-secretase activity was measured in each regions of brain at 7 day after treatment of LPS. β-secretase activity was presented by the unit of fluorescence which is proportional to enzyme activity. The effect of BV was tested in three independent experiments (n=30)

* Significantly different from positive LPS treated control ($p<0.05$).

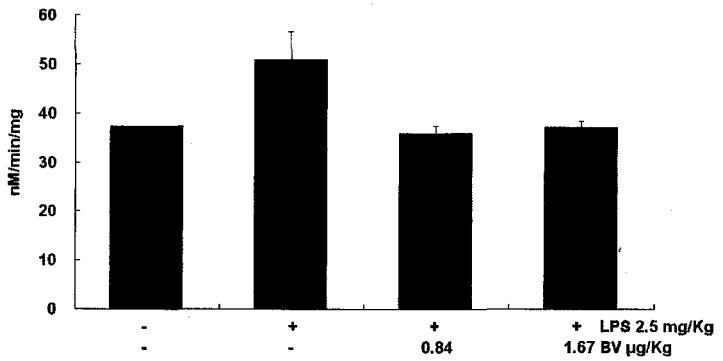


Fig. 11. The effect of BV on γ-secretase activity induced by LPS in cerebral cortex. BV were administrated by i.p., and then the mice were treated LPS (i.p.). The γ-secretase activity was measured in each regions of brain at 7 day after treatment of LPS. γ-secretase activity was presented by the unit of fluorescence which is proportional to enzyme activity. The effect of BV was tested in three independent experiments (n=30)

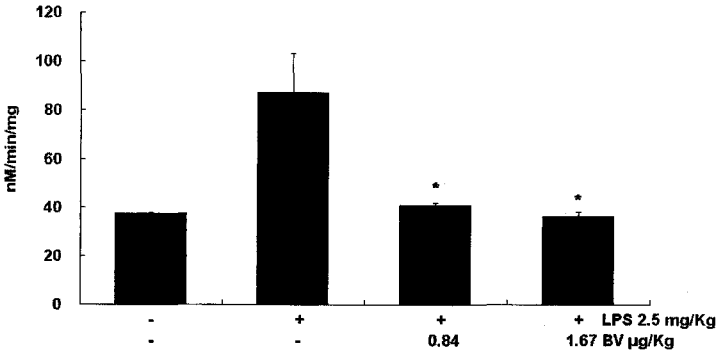


Fig. 12. The effect of BV on γ-secretase activity induced by LPS in hippocampus. BV were administrated by i.p., and then the mice were treated LPS (i.p.). The γ-secretase activity was measured in each regions of brain at 7 day after treatment of LPS. γ-secretase activity was presented by the unit of fluorescence which is proportional to enzyme activity. The effect of BV was tested in three independent experiments (n=30)

* Significantly different from positive LPS treated control ($p<0.05$).

Ⅳ. Discussion

Memorial impairment is known to be related with cholinergic nervous system. The lack of acetylcholine resulted from the degenerative change of the brain such as senile plaque is thought to be a major case of the memorial impairment, in which acetylcholine estrase, a breakdown enzyme of acetylcholine is involved[29-31].

Previous studies have shown that BV and its polyphenolic components possess neuroprotective effects[16-17,32-33]. Our study in here showed that BV reduced LPS-induced cognitive dysfunction in addition to the inhibitory effect on acetylcholine esterase and secretase activities. BV also has direct inhibition of Aβ aggregation. This data suggest that BV may have a role in ameliorating the AD related memory impairment. According to another previous studies, BV in vitro reduced Aβ 25-35-induced ROS generation which was accompanied by the inhibitory effect on Aβ 25-35-induced oxidative DNA damage and apoptotic neuronal cell death[34], and pretreatment of BV for

7 days at 1.5 and 3 mg/kg concentration reversed scopolamine (i.p. administration)-induced memorial deficit. Several lines of evidence have demonstrated that generation of ROS by Aβ is associated with the formation of senile plaques in the brains of AD patients which eventually cause neuronal cell death[35-36]. Previous other findings also showed that green tea extract protected from ischemia/reperfusion-induced memorial functions through anti-oxidative mechanism[16-17]. It is generally hypothesized that anti-oxidative mechanism of green tea or its component such as BV could be involved in its protective or preventive effects on several diseases or on toxic insults. Therefore it is possible that the improving effect of BV on memorial dysfunction induced LPS in the present study may be also related its anti-oxidative property.

In the present study, BV demonstrated anti-acetylcholine esterase and anti-secretase activity. In vivo inhibition of these enzymes were effective in maintaining the level of acetylcholine and reducing Aβ level, hence the activity could be pathophysiologically relevant with the recognition ability. In addition, I also found that BV has directly inhibiting effect on Aβ aggregation. Therefore, it can be suggested that inhibition of A β formation or/and aggregation of Aβ can serve as a neuroprotective effect against the toxicity of Aβ. It was also reported that green tea polyphenolic component BV increase soluble APP secretion through enhancing α form of secretase[37]. Soluble APP has neurotropic or neuroprotective activities[38]. These data therefore suggest that in addition to the anti-inflammatory and anti-oxidative properties, inhibitory effect on secretase activities and Aβ aggregation may be also important mechanisms of the preventing ability of BV on recognition dysfunction. The secretase inhibitors have been shown to reduce brain concentrations of Aβ in human APPV717F (PDAPP mice) transgenic mice and "Swedish" mutation ($APP_{K670N/M671L}$) Tg2576 mice[39].

Possible mechanism of how BV can inhibit secretase activity should be needed for further study. However, it is noteworthy that a secretory process of APP is dependent with the activation of the mitogen-activated protein (MAP) kinase pathway[40]. It was also observed that cytokine-elicited regulation of γ-secretase to control Aβ production by modulating JNK pathway[41]. The ability of BV or its component BV has been shown to suppresses MAP kinases in the LPS-induced phenotypic and functional maturation of murine dendritic[42], tumor promoter-induced MMP-9 expression in human gastric AGS cells[43]. It was also found that BV prevented apoptotic cell death through inhibition of MAP kinase activity in PC12 cells[34]. NF-κB pathway may be also involved in the inhibitory effect of BV on secretase activities. Therefore it is possible that inhibition of MAP kinase and/or NF-κB pathway can block secretase activity thereby reduce the formation of Aβ. I are currently investigating these possible mechanisms. In summary, our current study shows that BV has recovery effect against LPS-induced memory dysfunction through inhibition of acetylcholine esterase and secretase activity and Aβ aggregation in addition to anti-oxidation mechanism. This study therefore suggests that BV Acupuncture may be useful method for prevention of development or progression of AD.

V. References

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