The effects of bee venom pharmacopuncture on middle cerebral artery occlusion ischemic cerebral damage in mice

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ABSTRACT

Background: The therapeutic potential of Bee Venom Pharmacopuncture (BVP) on acute ischemic cerebral infraction was determined in mice in vivo and in vitro.

Methods: Analysis of acute ischemic cerebral infraction was performed using 7 week old male ICR mice (n = 20) and microglial BV-2 cells. Bee venom (5 µg/kg) was injected into the caudal vein of middle cerebral artery occlusion (MCAo) mice (1 hour after reperfusion, 3 hours after MCAo probe insertion), and also used to treat LPS-stimulated microglial BV-2 cells (1, 2, 5 µg/mL). Markers of inflammation were monitored.

Results: NO declined statistically significantly in BVP treated MCAo mice compared to the untreated MCAo group (p < 0.05). Compared to the MCAo group, the BVP-treated MCAo group showed a decreased production volume of malondialdehyde, but an increased glutathione/oxidized glutathione ratio. Compared to the untreated MCAo group, the BVP treated MCAo group showed a statistically significant decline in TNF and IL-1β levels (p < 0.05). BVP inhibited the levels of p65, p50, p-IκB-α, and levels of p-ERK1/2, p-JNK2, p-P38 declined.

Conclusion: BVP is effective at dampening the inflammatory response in vivo and in vitro and may supplement rt-PA treatment.

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Introduction

In 80% of cerebral stroke cases, ischemic cerebral infarction is the cause and 20% are caused by cerebral hemorrhage. Cerebral strokes are the largest cause of disability, the second largest cause of dementia, and the fourth largest cause of death in developed countries [1].

The only therapy for acute ischemic cerebral stroke has been intravenous (IV) administration of recombinant tissue plasminogen activator (rt-PA) [2] which induces reperfusion of occluded arteries, helping blood-borne leukocytes and cytokines to reach ischemic cerebral tissues [3]. However, it was reported that rt-PA needed to be administered within 3 to 4.5 hours following a cerebral stroke [4], and only 10% (approximately) of patients on the clinical trial were treated, implying limited usage of rt-PA [5]. It has also been reported that rt-PA therapy causes intracranial bleeding in 5% of the total number of patients [6]. In animal studies, rt-PA was effective at removing blood clots (thrombolytic) but it was neurotoxic, increasing neuronal damage caused by ischemia [7].

Due to the limitations of rt-PA, there has been research into drugs effective at protecting nerve cells and inhibiting apoptosis which is caused by a dysfunctional blood supply. Results from the clinical phase imply a limit to suggest a therapy which can replace or supplement rt-PA [8].

In Korean medicine, studies have been conducted to ascertain whether herbs/medicine are effective at neuroprotection in mice with cerebral ischemic damage. Studies on herbs that were effective at neuroprotection in animal studies include Sarijang, herb Angelica gigas Nakai, Semen Persicae, and Chungpyesagan-decotion [9-12]. These herbs were decocted and the concentrated extract was orally administered to mice.

Biotoxins can be defined as toxic substances produced from plants, animals and microorganisms. Biotoxins not only have deleterious toxic effects but also have beneficial effects in vivo. So far, many studies have shown anti-cancer effects of biotoxins in vivo/in vitro, due to cytotoxicity, immunomodulatory function, apoptotic effects, and anti-cancer activity [13].

In Korean medicine, bee venom pharmacopuncture (BVP) is

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performed when bee venom from the poison sac of the bee is extracted, processed and administered to acupuncture points and pressure points relevant to the disease by the principle of the meridian. BVP is widely used in various systemic and localized conditions like muscle pain, acute chronic arthritis, back pain, rheumatoid arthritis, shoulder inflammation and facial paralysis [14]. The effectiveness of BVP has been reported in the treatment after a stroke for hemiplegic shoulder pain [15,16]. In these case reports, BVP was used to treat the symptoms after a chronic cerebral infarction. There has been no any reports about the effectiveness of BVP on acute cerebral stroke. In an animal study, when phospholipase A2 (a component of bee venom) was injected into the middle cerebral artery occlusion (MCAo) a therapeutic effect on the cranial nerves was shown [17]. However, there has been no study to date on the mechanism of phospholipase A2 therapeutic effects on the nerves.

In 2015 it was reported that the cellular inflammatory response under brain ischemia (after blocking of cerebral blood vessels) induced brain tissue cell death [18]. Since inflammation during ischemia is a complex interaction between inflammatory cells and brain tissues, it was difficult to identify the immune-mechanisms after ischemic stroke. As the treatment time for reperfusion was short for blocked cerebral vasculature, the treatment aimed at modifying the inflammatory reaction which was effective at prolonging the entire treatment time and enhanced the treatment effects for ischemic brain damage. Therefore, studies on this were actively conducted [18].

BVP has been used for many inflammatory diseases in Korean medicine. Studies have identified that the immune-mechanism limits the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) by suppressing the expression of Nuclear factor-kB (NF-kB) and mitogen-activated protein kinases (MAPK), limiting the inflammatory reaction [19]. Modulating the inflammatory reaction to cephalin under acute ischemia may be an important treatment direction. Animal studies based on the assumption that BVP can impart substantial beneficial effects during acute cerebral infarction have shown it is effective in restraining inflammation.

The purpose of this study was to identify what impact BVP had on MCAo induced cerebral ischemia in mice and whether BVP improved neurological function. In addition, the impact of BVP on the inflammatory response to lipopolysaccharide (LPS) treated microglial BV-2 cell was also investigated. Furthermore the mechanism of inhibition of the inflammatory response induced by BVP was determined.

Materials and Methods

Materials and animals

I CR mice, bee venom and microglial BV-2 cells

Male ICR mice were purchased from Samtako Bio Korea Co. (Osan, Republic of Korea). BVP was purchased from You-Miel Bee Venom Ltd. (Hwasoon, Korea). The composition of the Bee Venom was as follows: 45-50% mellitin, 2.5-3% amine, 4-5% carbohydrate, and 19-27% of others, including protease inhibitor, glucosidase, invertase, acid phosphomonoesterase, dopamine, norepinephrine, and unknown amino acids, with 99.5% purity. The concentration of 5 µg/kg BVP was injected into the caudal vein of MCAo mice (1 hour after reperfusion, 3 hours after MCAo probe inserion). Microglial BV-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). To observe changes in microglial BV-2 cells depending on concentrations, different BVP concentrations of 1, 2, and 5 µg/mL were applied.

Reagents, antibodies

Anti-COX-2, anti-iKB, anti-p-IKB were purchased from Cell Signaling Mol Neurobiol Technology, Inc. (Beverly, MA, USA) and anti-iNOS from Novus Biologicals, Inc. (Littleton, CO, USA). Anti-p50, anti-p65, conjugated anti-mouse or anti-rabbit antibodies was purchased from Santa Cruz Biotehnologies Inc. (Santa Cruz, CA, USA). Antibodies against JNK, phosphor-JNK, p38, phosphor-p38 (p-P38), ERK, phosphor-ERK were purchased from Cell signaling Technology (Beverly, MA, USA) and anti-β-actin antibody from Sigma (St Louis, MO, USA). Lysis buffer for Western Blot was purchased from iNtron biotechnology (Sungnam, Korea) and nitrocellulose membrane from Amersham Pharmacia Biotech (Hybond ECL; Piscataway, NJ, USA). As a way to identify the production of reactive oxygen species (ROS), 2, 7-dichlorofluorescein diacetate (Sigma Aldrich, USA) was used. A lipid peroxidation assay kit (Cell Bioslabs, Inc. San Diego, CA, USA) was purchased and used to check the production of malondialdehyde (MDA), which is a compound of polyunsaturated fatty acids. Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) was used for cell culture.

Ischemia surgery

The acclimated 7-week-old ICR mice were anesthetized with a gas mixture of 75% N2O and 25% O2. The middle cerebral artery was occluded for 2 hours using sutures (Doccol Corp, Sharon, MA, USA). MCAo was performed on a total of 20 mice: 10 in BVP treated MCAo group, and 10 in untreated MCAo group, respectively.

The right common carotid artery and the right external carotid artery were exposed through a middle neck incision. The external carotid artery was dissected distally, ligated, and coagulated along with the terminal lingual and maxillary artery branches. A minimal incision was made in the external carotid artery stump, at an angle of incidence, with iridectomy scissors. Following the incision, occlusion was performed by sutures (0.22 mm). During the occlusion period and postoperative period (for 1 hour after MCA probe insertion), the animals were kept on thermostatically controlled warming plates in order to maintain body temperature at 37 °C to prevent hypothermia. Following the occlusion, clips were removed to restore blood for recirculation. The sham group underwent the same surgical procedure as MCAo group while limiting the incision depth to prevent cerebral artery occlusion (n = 10).

Assessment of neurological deficit score

Scores were evaluated for 2 consecutive days in the untreated MCAo group, BVP-treated MCAo group and sham group: on the day of MCA occlusion and BVP injection (1 hour after reperfusion), and 24 hours after MCAo probe insertion. Neurological deficits were evaluated as reported by Longa et al [20]. Scoring was based on 5 types of disorder: 1) failure to extend the left forepaw, 2) decreased grip strength of forepaw, 3) circling left by pulling the tail, 4) spontaneous circling, and 5) falling down. A rating of 1 point was given for each assessment, and then the scores were totaled up. Neurological function assessment was performed by an investigator blinded to the experimental groups.

Morphometric determination of infarction volume

The brain specimen was taken by sacrificing the ICR mice after 24 hours from the time the MCAo probe was inserted. For the detection of the ischemic infarction area of the brain, a
cross-sectional infarction area on the surfaces of each brain slice was defined by the 2, 3, 5-triphenyltetrazolium chloride (TTC) staining method. After 1 hour of reperfusion, the mice received an intracardiac perfusion of 0.9% buffered saline. The brain was then removed and cut into 2 mm serial slices starting 1 mm from the frontal pole. The coronal slices were then immersed in a 2% phosphate-buffered solution for 50 minutes at 37°C. After TTC staining, the slices were fixed in a 10% phosphate-buffered formalin, and the infarction area was then determined by an image analyzer using the Leica Qwin program (Leica Microsystems). The coronal slices were then immersed in 10% formalin, and the infarction area was then determined by an image program (Sion image, Scion Corporation, MD, USA), and then the infarct volume of the whole brain (mm³) was calculated by the sum of all the slices’ (7 slices in 1 brain) infarct areas volume × thickness (2 mm). The relative infarction volumes were indicated by the percentage of control brain infarction volume.

**Reactive oxygen species generation**

Generation of ROS was assessed by 2, 7-DCFH-DA (Sigma Aldrich, USA), an oxidation-sensitive fluorescent probe. Intracellular hydrogen peroxide (H₂O₂) or low-molecular-weight peroxides can oxidize 2, 7-dichlorofluorescein diacetate to the highly fluorescent compound dichlorofluorescein (DCF). Briefly, tissue lysates were plated in black 96 well plate and incubated with 10μM DCFH-DA at 37°C for 4 hours. Fluorescence intensity of DCF was measured in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

**Lipid peroxidation**

The formation of MDA, as a lipid peroxidation product in the whole brain homogenate of ipsilateral hemisphere (or left hemisphere of the sham-operated control), was determined using lipid peroxidation assay kit (Cell Biolabs, Inc. San Diego, CA, USA).

**Determination of nitric oxide in cerebrospinal fluid**

Cerebrospinal fluid was collected carefully so as not to be contaminated with blood by puncturing the cervical cisterna membrane after sacrificing the rats with ether anesthesia. Nitrite in cerebrospinal fluid was measured as a final product of nitric oxide (NO) generation using a Griess reagent system at 540 nm (Promega, Madison, WI, USA).

**Assay for glutathione peroxidase activity**

Six half brains were used to assay Glutathione (GSH) peroxidase activity. Brains were taken by sacrificing the ICR mice after 72 hours of MCAo (insert of MCAo probe). Brain tissues were homogenized with lysis buffer (PROPREP; iNTRON, Sungnam, Republic of Korea; n = 6 mice per group) and centrifuged at 2,500 g for 15 minutes at 4°C. Equal amounts of total protein (40 μg) isolated from brain tissues were resolved on 8% or 10% sodium dodecyl sulfate polyacrylamide gels and then transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were incubated at room temperature for 2 hours with the following specific antibodies: anti-COX-2, anti-IκB, anti-p-IκB (Cell Signaling Mol Neurobiol Technology, Inc., Beverly, MA, USA), anti-iNOS (1:1,000 Novus Biologicals, Inc., Littleton, CO, USA), anti-p50, anti-p65, (1:1,000; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA), Mouse monoclonal antibodies directed against JNK, phosphor-JNK, p38, p-P38, ERK, phosphor-ERK (1:1,000) (Cell signaling Technology, Beverly, MA, USA) and anti-β-actin (1:2,500; Sigma, St Louis, MO, USA). Blots were then incubated at room temperature for 2 hours with, corresponding peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:2,000; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection system. The relative density of the protein bands was scanned densitometrically using My Image (SLB, Seoul, Korea) and quantified by Lab Works 4.0 (UVP, Upland, CA, USA).

**Levels of interleukin-1β and tumor necrosis factor-α in serum**

The levels of interleukin-1β (IL-1β) and TNF (tumor necrosis factor) in serum were measured by a sandwich enzyme-linked immunosorbent assay according to the instructions of the manufacturer. Briefly, dispersed antigen standards and samples were added to each well of 96-well plates pre-coated with first antibodies. After adding Biotin Conjugate Reagent and Enzyme Conjugate Reagent into each well, the plates were incubated at 37°C for 60 minutes. Then the plates were rinsed 5 × with distilled water. After the chromogenic reaction, microtiter plate reader was performed within 30 minutes measuring the absorbance at 405 nm. The IL-1β and TNF levels in the blood were expressed as picogram of cytokines per milliliter of serum.

**Nitrite quantification assay**

NO was determined through the detection of nitrite levels in the cell culture medium. The microglial BV-2 cells were seeded in 6-well plates (1 × 106 cells/well) with 2 mL of cell culture medium and incubated for 24 hours. This was followed by discarding the old culture medium and replacing it with the new medium to maintain the cells. Different concentrations of BVP at various concentrations (1, 2, or 5 μg/mL) were pretreated with Microglial BV-2 cells. Induction of Microglial BV-2 cells with LPS (1 μg/mL) for all samples was conducted except for the control for another 24 hours. Then, 100 μL of the collected supernatants was added with 100 μL of Griess reagent (0.1% NED, 1% sulphanilamide, and 2.5% phosphoric acid) and incubated at room temperature for 10 minutes in the dark. The absorbance was determined by using a microplate reader at 540 nm wavelength. The NO concentration was determined by comparison to the standard curve.
Cell culture

Microglial BV-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were maintained at subconfluence in 95% air, 5% CO₂ humidified atmosphere at 37°C. The medium used for routine subcultivation was Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL).

Cells were incubated with BVP at various concentrations (1, 2, or 5 µg/mL) or positive chemicals and then stimulated with LPS 1 µg/mL for the indicated time in figure legends. Various concentrations of BVP dissolved in dimethylsulfoxide (DMSO)-ethanol solvent (1:1 DMSO: ethanol, v/v) were added together with LPS. The final concentration of DMSO-ethanol solvent used was less than 0.05%. Cells were treated with 0.05% DMSO-ethanol solvent as vehicle control (Sham group).

Statistical analysis

The data were analyzed using the GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA). Data were presented as mean ± SD. Differences in the data were assessed by a one-way analysis of variance (ANOVA). When the p value in the ANOVA test indicated statistical significance, the differences were assessed by the Dunnett’s test. A value of p < 0.05 was considered to be statistically significant.

Results

Effect of BVP on brain infarction induced by MCAo in mice

The potential therapeutic effects of BVP in MCAo induced mice was investigated and it was determined that the volume of infarction in the BVP treated MCAo group was statistically significantly decreased when compared with the untreated MCAo group (Fig. 1).

Effect of BVP on neurological behavior improvement in MCAo induced brain infarction in mice

The potential therapeutic effects of BVP on neurological behavior in mice was investigated. The neurological deficit score was reduced in the BVP treated MCAo group compared with the untreated MCAo group and this observation was time-dependent, where the score was statistically significantly lower in the BVP treated MCAo group measured a day after the experiment, compared with the experiment day. No improvement was observed in the untreated MCAo group (Fig. 2).

Effect of BVP on H₂O₂ production in MCAo induced brain infarction in mice

To investigate the amount of ROS produced, the H₂O₂ concentrations among the Sham group, untreated MCAo group and BVP treated MCAo group were compared. H₂O₂ is a type of ROS, and the higher the ROS concentration, the more likely it is that apoptosis will occur. As shown in Fig. 3, the H₂O₂ concentration was statistically significantly lower in the BVP treated MCAo group (5 µg/kg, IV) when compared with the untreated MCAo group, implying that BVP inhibited the formation of ROS in the brain cells.

Fig. 1. Effect of BVP on the brain infarction induced by MCAo. Ischemic mice were prepared by transient middle cerebral artery occlusion at 2 h (120 minutes) and reperfusion (60 minutes). (A) Brain infarction was determined by TTC staining. Normal area in brain section is stained in dark pink, and infarcted area in white. (B) Infarct volumes in the untreated MCAo group and the BVP treated MCAo group are expressed as the percentage of volumes, and presented as the means ± SD (column: means, bar: SD; ***p < 0.001). BVP, bee venom pharmacopuncture; MCAo, middle cerebral artery occlusion.

Fig. 2. Effect of BVP on neurological behavior improvement in the brain infarcted mice induced by MCAo. The degree of improvement in neurological behavior was compared between the untreated MCAo group and the BVP treated MCAo group. The comparison was assessed based on the Neurological deficits score and evaluated by an investigator who was unaware of the BVP treatment. (A) Evaluation standard of Neurological deficits score and evaluated by an investigator who was unaware of the BVP treatment. (B) The result was normal in the Sham group (no MCAo), whereas in MCAo group, a deficit of function according to the cerebral ischemia was observed. But the score was low in the BVP treated MCAo group. The untreated MCAo group, hardly showed an improvement in neurological behavior even after a day had passed, whereas the BVP treated MCAo group showed a significant improvement in neurological behavior. It is presented as the means ± SD (column: means, bar: SD; **p < 0.01). BVP, bee venom pharmacopuncture; MCAo, middle cerebral artery occlusion.

Fig. 3. Effect of BVP on H₂O₂ production in MCAo induced brain infarction in mice. The H₂O₂ concentrations among the Sham group, untreated MCAo group and BVP treated MCAo group were compared. H₂O₂ is a type of ROS, and the higher the ROS concentration, the more likely it is that apoptosis will occur. As shown in Fig. 3, the H₂O₂ concentration was statistically significantly lower in the BVP treated MCAo group (5 µg/kg, IV) when compared with the untreated MCAo group, implying that BVP inhibited the formation of ROS in the brain cells.
IV), and was statistically significantly higher in the BVP treated group (5 µg/kg, IV). The concentration was reduced in the BVP treated MCAo group than the left hemisphere of the Sham group, which indicated that the concentration of MDA in the untreated MCAo group was higher than in the left hemisphere of the sham group. As shown in Fig. 5, the concentration was significantly lower in the BVP treated MCAo group (5 µg/kg I.V) than the untreated MCAo group. It is presented as the means ± SD (column: means, bar: SD, **: p < 0.01).

BVP, bee venom pharmacopuncture; MCAo, middle cerebral artery occlusion.

Effect of BVP on NO production in MCAo induced brain infarction in mice

The concentration of NO was compared among the Sham group, untreated MCAo group, and BVP treated MCAo group and showed that the NO level was higher in the untreated MCAo group than the Sham group. The NO level was statistically significantly decreased in the BVP with MCAo group (5 µg/kg, IV; Fig. 4).

Effect of BVP on MDA concentration (result of lipid peroxidation) in MCAo induced brain infarction in mice

The concentration of MDA, the lipid peroxidation product was measured in the hemisphere homogenate, or the homogenate from the brain infarction induced by MCAo (Unit: μm). The concentration of malonaldehyde (lipid peroxidation product) was analyzed using a lipid peroxidation (TBARS) assay kit. The concentration of malonaldehyde was higher in the untreated MCAo group than in the Sham group, and the concentration was lower in the BVP treated MCAo group (5 µg/kg LV) than the untreated MCAo group. It is presented as the means ± SD (column: means, bar: SD). BVP, bee venom pharmacopuncture; MCAo, middle cerebral artery occlusion.

Effect of BVP on glutathione /oxidized glutathione ratio in MCAo induced brain infarction in mice

The GSH/GSSG ratio was compared among the Sham group, untreated MCAo group, and BVP treated MCAo group (5 µg/kg, IV), and was statistically significantly higher in the BVP treated MCAo group compared with the untreated MCAo group (Fig. 6).

Effect of BVP on tumor necrosis factor and IL-1β levels in MCAo induced brain infarction in mice

The serum concentrations (pg/mL) of IL-1β and TNF were measured and compared among the Sham group, untreated MCAo group, and BVP treated MCAo group (5 µg/kg, IV), and the concentrations of IL-1β and TNF in the untreated MCAo group were statistically significantly lower than the untreated MCAo group (Fig. 7).
Effect of BVP on expression of inducible iNOS, COX-2 proteins in MCAo induced brain infarction in mice

The effect of BVP on the expression of iNOS and COX-2, the inflammation-promoting factors was investigated by Western blotting. As shown in Fig. 8, the level of expression of β-actin was similar in all groups. The expression level of iNOS and COX-2 increased in the untreated MCAo group when compared with the Sham group. Moreover, iNOS and COX-2 protein expression levels were decreased in the BVP treated MCAo group (5 µg/kg, IV), compared with the untreated MCAo group.

Effect of BVP on expression of p65, p50 in MCAo induced brain infarction in mice

The amount of p50 and p65 within the nucleus was measured to investigate the effect of BVP in the activation of NF-κB involved in the control of proinflammatory cytokines and mediators. As shown in Fig. 9, the level of expression of Histone H1 was similar in all groups. As shown in Fig. 10, the level of expression of p-IκB-α was lower in the BVP treated group compared with the untreated group. Moreover, p50 and p65 protein expression levels were decreased in the BVP treated MCAo group (5 µg/kg, IV), compared with the untreated MCAo group.

Effect of BVP on expression of MAPKs (JNK, ERK, P-38) MCAo induced brain infarction

The effect of BVP on the expression of MAPKs in stroke-induced MCAo was investigated. The expression level of p-ERK increased by half in the untreated MCAo group compared with the Sham group, indicating that ERK phosphorylation was active in the untreated MCAo group. Moreover, in BVP treated MCAo group (5 µg/kg, IV), p-ERK expression decreased when compared with the untreated MCAo group, which implied that BVP could inhibit the activation of NF-κB by inhibiting the phosphorylation of p-ERK.

Effect of BVP on expression of p-IκB-α, phosphorylated p-IκB-α proteins in MCAo induced brain infarction in mice

The expression level of p-IκB-α was similar in all groups. The expression of p-IκB-α was increased in the untreated MCAo group compared with the Sham group, indicating that IκB phosphorylation was active in the untreated MCAo group. Moreover, in BVP treated MCAo group (5 µg/kg, IV), p-IκB-α expression decreased when compared with the untreated MCAo group, which implied that BVP could inhibit the activation of NF-κB by inhibiting the phosphorylation of IκB-α.
**Effect of BVP on NO production in LPS stimulated Microglial BV-2 cells**

Microglial BV-2 cells were pretreated with BVP (0, 1, 2, 5 μg/mL) and with LPS, and the suppressing effect of BVP on NO (induced by iNOS) was evaluated. As shown in Fig. 12, the NO concentration was higher in the LPS-treated group than the sham group, which was reduced in the BVP + LPS treated group. It was difficult to say that it had decreased in a concentration-dependent manner.

**Effects of BVP on H₂O₂ production in LPS stimulated Microglial BV-2 cells**

The effects of BVP concentration (0, 1, 2, 5 μg/mL) on the production of H₂O₂ produced by Microglial BV-2 cells when stimulated with LPS was investigated. As shown in Fig. 13, the concentration of H₂O₂ was higher in the LPS treated group than the sham group, which was reduced in the BVP + LPS treated group. Although it was difficult to say that it decreased in a concentration-dependent manner, the concentration of H₂O₂ in the 5 μg/mL BVP + LPS treated group was similar to the Sham group.

**Effects of BVP on iNOS or COX-2 in LPS stimulated Microglial BV-2 cells**

The effects of BVP concentration (0, 1, 2, 5 μg/mL) on the expression of iNOS or COX-2 in LPS stimulated Microglial BV-2 cells was investigated. Western blot was used to analyze the expression levels of iNOS, COX-2. For above blotting, β-actin was used as a control. Each band is representative for 3 experiments.

**Effects of BVP on the expression of p65 or p50 in LPS stimulated Microglial BV-2 cells**

The expression of p65 or p50 in LPS stimulated Microglial BV-2 cells. Microglial BV-2 cells were pre-treated with BVP at various concentrations (1, 2, 5 μg/mL) for 3 hours, followed by stimulation with LPS for 24 hours. The expression levels of p65 and p50 were analyzed by Western blotting. Each band is representative for 3 experiments.
Microglial cells are specialized macrophages found in the tissues of the CNS and act as the primary immune responder to acute neuronal and CNS damage. Once microglial cells are activated, they proliferate and migrate to the site of injury and release cytotoxic and/or inflammatory mediators like NO, TNF, MCP-1, MIP-1α, IL-1β, IL-6, IL-8 and cytokines. Such cerebral inflammatory reactions are related to neurodegenerative diseases including multiple sclerosis, Alzheimer’s and Parkinson’s [23]. In murine studies of ischemic cerebral infarction TNF and IL-1 have been reported to be produced by microglial cells [24]. Transient focal cerebral ischemia can be as worse as severe ischemia within 2 weeks, and it is attributable to apoptosis of neurons induced by active protein synthesis which occurs after ischemia [25]. LPS exists on the cell wall of gram-negative bacterium and in vivo produce endotoxins that infect the blood, stimulating microglial cells to trigger an inflammatory reaction in the cerebrum which has a neuroprotective effect [26,27]. In this current study, the response of microglial cell to LPS was used to determine the mechanism involved when BVP inhibits an inflammatory reaction. Following MCA occlusion, BVP (5 µg/kg) treated group. In the BVP 5 µg/mL high concentration group, the expression of p-IκB-α was lower than the Sham group (without LPS treatment). This result implied that BVP may inhibit the activation of NF-κB by suppressing IκB-α phosphorylation.

**Effects of BVP on the expression of MAPKs (JNK, ERK, p38) in LPS stimulated Microglial BV-2 cells**

The effects of BVP concentration (0, 1, 2, 5 µg/mL) on MAPKs expression associated with NF-κB activation was investigated. The expression levels of JNK, p-JNK2, ERK, p-ERK 1/2, p38, and p-P38 increased in the LPS treated group compared with the sham group. This result suggested that LPS treatment induced inflammation thereby increasing overall MAPKs expression. The expression levels of p-JNK2, p-ERK 1/2, and p-P38 in the BVP + LPS treated group were statistically significantly decreased. This suggested that BVP may inhibit the activation of NF-κB by suppressing the phosphorylation of JNK, ERK, and p38 (Fig. 17).

**Discussion**

Ischemic cerebral infarction is a common cause of strokes, accounting for 80% with 1 in 7 people predicted to have a stroke during their life time. Cerebral infarction if not fatal, is the cause of chronic physical disability [1]. Administration of rt-PA within 3 to 4.5 hours after a stroke allows reperfusion of occluded artery [4]. Any treatments that can work with the restrictive time frame for rt-PA administration or reduce any toxicity that may occur would be beneficial. The murine MCAo model of ischemic stroke is a reliable model compared with other models (Craniotomy model, Photothermolysis model, Endothelin-1 model) [21].

The purpose of the study was to observe the effects of BVP performed in a murine model at the beginning of ischemic cerebral infarction and determine the mechanisms involved. The study was largely divided into 2 parts. The first part was to analyze the cerebral damage and observe neurological behavioral improvements between the BVP treated group, which had acute cerebral ischemia after MCA occlusion, and the sham group. Immunohistochemical examination was conducted to determine which mechanisms were involved in neuroinflammation and apoptosis. The second part of the study was based on LPS-stimulated microglial BV-2 cells to identify whether BVP inhibited inflammation, and determine which mechanisms were involved.

Microglial cells are specialized macrophages found in the tissues of the CNS and act as the primary immune responder to acute neuronal and CNS damage. Once microglial cells are activated, they proliferate and migrate to the site of injury and release cytotoxic and/or inflammatory mediators [22] like NO, TNF, MCP-1, MIP-1α, IL-1β, IL-6, IL-8 and cytokines. Such cerebral inflammatory reactions are related to neurodegenerative diseases including multiple sclerosis, Alzheimer’s and Parkinson’s [23]. In murine studies of ischemic cerebral infarction TNF and IL-1 have been reported to be produced by microglial cells [24]. Transient focal cerebral ischemia can be as worse as severe ischemia within 2 weeks, and it is attributable to apoptosis of neurons induced by active protein synthesis which occurs after ischemia [25].

**Effects of BVP on the expression of p-IκB-α and IκB-α in LPS stimulated Microglial BV-2 cells**

The effects of BVP concentration (0, 1, 2, 5 µg/mL) on the presence of IκB-α and p-IκB-α within the nucleus which is associated with NF-κB activation, was investigated. As shown in Fig. 16, p-IκB-α increased in the LPS-treated group compared with the sham group. This implied that the phosphorylation of IκB-α was actively carried out following LPS treatment. Moreover, p-IκB-α expression decreased proportionally to the concentration of BVP in the BVP + LPS treated group compared with the LPS

Although inhibition of p65 expression was hardly observed in the BVP + LPS treated group, the expression level of p50 decreased in a concentration-dependent manner. It can be assumed that BVP may inhibit p50 expression in LPS stimulated microglial BV-2 cells.

**Effects of BVP on the expression of p-IκB-α and IκB-α in LPS stimulated Microglial BV-2 cells**

The effects of BVP concentration (1, 2, 5 µg/mL) for 3 hours, followed by stimulation with LPS for 24 hours. The expression levels of p-IκB-α and IκB-α were analyzed using Western blot. BVP, bee venom pharmacopuncture; LPS, lipopolysaccharide.

**Effects of BVP on the expression of MAPKs (JNK, ERK, p38) in LPS stimulated Microglial BV-2 cells**

The effects of BVP concentration (0, 1, 2, 5 µg/mL) for 3 hours, followed by stimulation with LPS for 24 hours. The expression levels of JNK, p-JNK2, ERK, p-ERK 1/2, p38, and p-P38 were analyzed by Western blotting. At this time, β-actin with the least difference in expression level was used as a control. Each band is representative for 3 experiments. BVP, bee venom pharmacopuncture; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPKs, mitogen activated protein kinase.
was administered by IV to mice which induced ischemic change in the brain cells and TTC staining allowed the ischemic cerebral area to be observed to identify a statistically significant decline in infarct volume. Such a decrease in infarct volume leads to neurological behavioral improvement, consequently reducing the neurological deficit score. Such behavior improvement becomes visible 1 day after BVP treatment (Fig. 2). The MCAo model is reliable and shows a positive correlation between infarction volume, neurological scores, and behavioral deficits [28]. This implies that when BVP is administered by IV, it may improve cerebral ischemia and consequently the neurological symptoms of a stroke.

Another aspect of the study was to identify the mechanism that BVP used to inhibit ischemic cerebral damage and whether treatment with bee venom leads to neurological behavioral improvement. At the onset of cerebral ischemia, the concentrations of ROS and NO has been reported to increase. ROS and NO induce necrosis, neuroinflammation and occlusion of normal neurons, consequently limiting memory and motor functions [29]. In this current study, the concentration level of NO and H₂O₂ was lower in BVP treated MCAo group (5 µg/kg) than in the untreated MCAo group. BVP was also shown to reduce the concentration level of H₂O₂ and NO in microglial BV-2 cells and were able to induce an acute inflammatory reaction to LPS (Figs. 12 and 13). This implied that BVP inhibited the production of substances which induce apoptosis and neuroinflammation, and may prevent neuronal occlusion which would improve neurological behavior.

NO is produced by NO synthases when iNOS has been activated during cerebral ischemia worsening the state in the penumbra [30,31]. In this study the production of iNOS was studied in the BVP treated MCAo group, the untreated MCAo group (Fig. 8), LPS-stimulated BVP treated microglial BV-2 cells (Fig. 14), and the sham group. In vivo and in vitro, the BVP treated group showed lower levels of iNOS compared with the untreated group, which appeared to be associated with the decline in NO concentration.

The concentration of MDA, a product of lipid peroxidation, was analyzed with a lipid peroxidation assay kit in the homogenate of the brain hemisphere, or homogenate of left hemisphere of sham-operated controls. It showed a lower concentration of MDA in the BVP treated MCAo group compared to the untreated MCAo group (Fig. 5). MDA is a product of lipid peroxidation by free radicals and it is also an indicator of the process of lipid peroxidation [32]. Consequently, it implies that BVP is involved in limiting the production of free radicals, inhibiting lipid peroxidation.

GSH is a substance which stabilizes free radicals and inhibits lipid peroxidation. The ratio of GSH to oxidised GSH (GSSG) is used as an index of oxidative stress and the health of the cell. GSH/GSSG ratio is high among normal cells but it decreases in neurodegenerative diseases like Alzheimer’s and Parkinson’s. Comparing the GSH/GSSG ratio in an experimental model and sham group using histopathology, allows the assessment efficacy of the potential therapy [33,34]. Compared with the untreated MCAo group, the GSH/GSSG ratio was higher in the BVP treated MCAo group but the ratio increased similarly to the level in the sham group (non-MCAo), which implied that BVP may contribute to the stabilization of free radicals, restoring cellular functions to normal.

In this study the concentration levels of TNF and IL-1β were statistically significantly reduced in the BVP treated MCAo group (p < 0.05; Fig. 7). It has been reported that the level of IL-1β and TNF increases at the onset of acute ischemic cerebral infarction [35] during the acute inflammatory response [36]. In murine studies of cerebral infarction, IL-1β and TNF has been reported to be produced mainly by microglial cells [24]. TNF has been shown to be both neurotoxic and neuroprotective, however, IL-1β has a neurotoxic effect [3]. BVP may inhibit the occurrence of free radical and NO in microglial cells, containing the acute immune response and reducing IL-1β and TNF levels.

COX exists in vivo as 2 forms; COX-1 and COX-2 which are different in terms of physiological and pathological functions and distribution systems. When LPS or inflammatory cytokines such as IL-1, IL-6, and TNF are released, COX-2 is activated [37]. COX-2 is complex because it is observed in normal brain tissue and contributes to the normal function of the brain [38]. However, during an inflammatory reaction like cerebral ischemia, COX-2 is rapidly produced, which is known to worsen brain damage [39]. One of the reasons for increased levels of COX-2 during acute cerebral ischemia is the production of inflammatory cytokines which increase the pressure and edema inside the cerebrum [38].

The concentrations of COX-2 were compared in vivo between the BVP treated group where acute ischemic cerebral damage occurred after MCA occlusion, with the sham group (Fig. 8), and in vitro with the LPS-stimulated BVP treated microglial BV-2 cells (Fig. 14). The result showed that the concentration of COX-2 was lower in the BVP treated MCAo group compared with the untreated MCAo group. It may indicate that BVP inhibited inflammatory cytokines like IL-1β and TNF, reducing the production of COX-2.

During inflammatory reactions NO, prostaglandin E₂, and interleukins are produced. ROS, NO and prostaglandin E₂ are induced by iNOS and COX-2. NF-kb is a crucial transcription factor related to the control of proinflammatory cytokines and mediators. Pathway of NF-kb activation can be divided into classic and alternative pathway. In the classical pathway, NF-kb dimers which are bound to IkBs exist in the cytoplasm, as p65, p50 subunits. When cells are stimulated by proinflammatory factors, IkBs becomes phosphorylated by IkB kinase and is dissociated. Free NF-kb activates gene transcription to produce proinflammatory mediators (chemokines and inflammatory cytokines, like TNF, IL-1β, and IL-6) [40,41]. Thus, the activation of the NF-kb pathway can be assessed by analyzing the occurrence of p65, p50, IkB-a and manifestation of p-IkB-a. In this study, the occurrence of p65, p50, and IkB-a declined in the BVP treated MCAo group compared with the untreated MCAo group (Figs. 9 and 10). A similar result was observed in LPS-stimulated BVP treated microglial BV-2 cells (Figs. 15 and 16). This implied that BVP may inhibit the inflammatory reaction caused by cerebral ischemia, through an inhibition of inflammatory activation in microglial cells.

The MAPK cascade involves a series of phosphoenzymes that are involved in cellular proliferation, cellular differentiation, inflammatory reaction and apoptosis, and when protein kinases become phosphorylated and activated. At last stage, it moved to nucleus and transduced as a response to extracellular signals. There are 3 kinds of MAPK families: classical MAPK (ERK), stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), and p38 kinase [42]. In this study, the amount of p-ERK was reduced by 1/2 in the BVP treated MCAo group compared with the untreated MCAo group, but there was no statistically significant difference between the levels of p-P38 and p-JNK2 in the BVP treated MCAo group and the untreated MCAo group (Fig. 11). In addition, p-JNK2, p-ERK 1/2, p-P38 were reduced in LPS stimulated BVP treated microglial BV-2 cells compared with the LPS stimulated microglial BV-2 cells (Fig. 17). This implied that the MAPK cascade was involved in BVP apparent containment of the inflammatory reaction, leading to the presumption that BVP may be effective in containing classical MAPK (ERK) reactions among 3 MAPK family members. It was also assumed that BVP...
may contain MAPK, SAPK/JNK, p38 kinase because of the observations in vitro in microglial cells, but when acute ischemic cerebral damage occurs due to MCAo, bee venom may contain the classical MAPK (ERK) pathway effectively.

In this study, the MCAo model was used where the reperfusion time was shorter than in the clinical setting, and the age was young compared with stroke patients who are older and have many risk factors. More studies are required to identify the optimal injection volume of bee venom and in what form it will be used and excreted, and whether there are inherent risk factors in its use [21]. However, in this study, BVP was shown to inhibit the NF-kB and MAPK pathway by limiting the activation of microglial cell production of inflammatory cytokines and reducing the production free radicals and NO, consequently reducing the ischemic damage in cerebral cells of mice. Based on these results BVP may be utilized as an effective neuroprotective medicine in the clinic.

Conflicts of Interest

The authors have no conflicts of interest to declare.

References