

# Effect of modified Bo-yang-Hwan-o-Tang, a polyherbal medicine on the hippocampal neuronal damage in a rat model of global ischemia

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## ABSTRACT

**Background:** Chronic cerebral hypoperfusion has been well-characterized as a common pathological status contributing to vascular dementia (VD). In this study, the neuroprotective effect of modified Bo-yang-Hwan-O-Tang (mBHT), a polyherbal medicine for ischemic stroke, was investigated in a rat model for global ischemia. **Materials and Methods:** Global ischemia model was prepared in Sprague-Dawley rats by the permanent occlusion of bilateral common carotid arteries (two-vessel occlusion [2VO])-induced chronic cerebral hypoperfusion. mBHT at doses of 250 and 500 mg/kg was orally administrated for 4 weeks once a day, 24 h after 2VO. Histopathological change of the hippocampal region was observed by hematoxylin and eosin, Nissl, and Fluoro-Jade B staining and immunohistochemistry with anti-gliial fibrillary acidic protein and anti-neuronal nuclei antibodies. The expression of Bax, Bcl-2, and caspase-3 was investigated in the hippocampus by Western blot. The nuclear factor-kappa B (NF-κB) expression was also analyzed in hippocampal CA1 region using immunofluorescence staining. **Results:** The administration of mBHT at doses of 250 and 500 mg/kg significantly inhibited chronic cerebral hypoperfusion-induced neuronal damage and astroglial activation in the hippocampal CA1 region in 2VO rats. mBHT increased the NF-κB expression in the CA1 neuronal cells but decreased in activated astrocytes. In addition, mBHT significantly decreased the hippocampal expression of Bax and caspase-3 and increased the Bcl-2 expression in 2VO rats. **Conclusions:** Our data indicate that mBHT has a neuroprotective property in VD induced by chronic cerebral hypoperfusion through inhibiting the hippocampal neuronal damage and astrogliosis.

**Key words:** Chronic cerebral hypoperfusion, hippocampus, modified Boyang-Hwan-o-Tang, neuroprotection, polyherbal medicine, two-vessel occlusion, vascular dementia

## INTRODUCTION

Recently, vascular dementia (VD) is considered to be an important problem due to their potential severity and increasing prevalence. VD is caused by problems in the blood supply to the brain, typically by a series of strokes and may contribute to the progression of neurodegenerative diseases. Patients suffering from VD present with cognitive impairment after an acute cerebrovascular event and develop progressive cognitive, motor and behavioral signs and symptoms.<sup>[1]</sup> The condition of VD is not a single

disease, but rather multifactorial conditions with different pathophysiological mechanisms such as ischemia or hemorrhage secondary to cerebrovascular diseases.<sup>[2,3]</sup> In cerebral ischemia, it produces abnormal levels of glucose, cholinergic substances, reactive oxygen species, and other metabolic substances, which may initiate and sustain the cascade of neuropathological events underlying VD.<sup>[4]</sup> Eventually, cerebral circulation disturbances in ischemia have been related with a decline in cognitive function in elderly subjects and development of vascular.<sup>[5]</sup>

Many recent therapeutic options for incurable brain diseases with multifactorial mechanisms such as cerebral ischemia, VD, and neurodegenerative disease have focused on a synergistic combination of ingredients rather than monotherapy.<sup>[6]</sup> Traditional medicines including Traditional Korean Medicine (TKM) have long been used to treat diverse

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human diseases including brain diseases and to maintain good health. The search for appropriate neuroprotective agents has focused on these traditional medicines because of leads provided by natural products that may be better treatments for brain diseases than currently used drugs.<sup>[7]</sup> Modified Bo-Yang-Hwan-O-Tang (mBHT) is a polyherbal medicine which comes from the prescription of TKM used in stroke, VD, senile, and heart damage,<sup>[8,9]</sup> and is newly developing as a natural drug for treatment of the patients with ischemic stroke. mBHT is comprised of twelve different herbs,<sup>[10]</sup> and previously reported its various effects such as vasodilation,<sup>[10]</sup> neuroprotection,<sup>[11]</sup> antioxidation,<sup>[12]</sup> antiinflammation,<sup>[13]</sup> and the amelioration of brain infarction, and edema and the neurological deficit by cerebral ischemia in rats.<sup>[14]</sup>

Therefore, the present study investigated the therapeutic benefit of mBHT on VD through the cognition enhancing, and brain damage amelioration in two-vessel occlusion (2VO) rats using the histopathological examination, and observation of neuronal cell death, and astroglial cell activation. However, the effect of mBHT on chronic cerebral hypoperfusion-induced VD has been firstly reported in here.

## MATERIALS AND METHODS

### Preparation of modified Bo-yang-Hwan-O-Tang

Modified Bo-yang-Hwan-O-Tang was composed of the 12 herbs described previously.<sup>[11-14]</sup> Each herb was purchased from Medicinal Materials Company (Kwangmyungdang Medicinal Herbs, Ulsan, Republic of Korea). All herbs were authenticated by Professor J-H. Lee, a medical botanist in the Department of Herbology, College of Korean Medicine, Dongguk University (DUCOM), Republic of Korea. Voucher specimens were deposited in the Herbarium of the DUCOM under registration number 08001C. A mixture of the 12 herbs was prepared by following the standard procedure in the pharmaceutical company (Hanpoong Pharm. Co Ltd., Seoul, Republic of Korea). In brief, each of the dried herbs was mixed together (50 kg in sum) and the ground to small pieces. The mixture was boiled in purified drinking water for 3 h, filtered through a two-layer mesh and concentrated under vacuum pressure at 700 mmHg for 15 h, after which it was freeze-dried to yield 34.8%. The lyophilized powder of mBHT was then stored at 4°C until use, at which time it was dissolved in saline.

### Animals

Male Sprague-Dawley rats (Orient Bio Inc., Gyeonggi-do, Rep. of Korea) weighting  $280 \pm 10$  g were used in all experiments. Rats were housed at ambient temperature of  $23^\circ\text{C} \pm 1^\circ\text{C}$ , relative humidity of  $50\% \pm 10\%$  and 12 h

light/dark cycle with free access to food and water. Rats were handled according to the animal welfare guidelines issued by the Korean National Institute of Health and the Korean Academy of Medical Sciences for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the Dongguk University.

### Preparation of ischemic stroke *in vivo* model

Rats were subjected to 2VO surgery as described previously.<sup>[4]</sup> Briefly, rats were anesthetized with 4% isoflurane and maintained using 1% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide during the surgical process. A midline incision was made, and both common carotid arteries were exposed; care was taken to avoid the vagus nerves. The carotid arteries were double ligated using 4-0 silk sutures. With the exception of occlusion of the carotid arteries, surgical procedures in sham-operated animals were the same as those in the bilateral common carotid artery-occluded (BCCAO)-operated animals. During surgery, rectal temperature was maintained at  $37.0\text{--}37.9^\circ\text{C}$  with a heating pad (FHC Inc., ME, USA). After the operation, all animals were returned to their cages with free access to food and water. On the 30<sup>th</sup> day after surgery (BCCAO induction or sham), the animals were drug treatment. Body weight (bw) was measured before the surgery and on the day of euthanasia.

### Drug treatment

Chronic cerebral hypoperfusion can be induced by permanent, BCCAO in rats, resulting in significant white matter lesions, learning and memory impairment, and hippocampal neuronal damage. After 30 days postsurgery, rats which were confirmed the induction of disease onset using water maze test randomly divided as follows: Rats were randomly divided into four groups: 2VO rats treated with saline, and 2VO rats treated with mBHT at doses of 50, 100, 250, and 500 mg/kg bw. The sham-operated rats were treated with saline instead of mBHT administration had been stopped for 2 weeks (from day 31 to day 42 postsurgery). Each group consisted of six rats with identical mean bws. Daily oral treatment of mBHT or vehicle (saline) started on day 30 postpermanent occlusion, and lasted for the termination of the experiment on 42 day.

### Histopathological analysis

On day 31, all rats in each group were deeply anesthetized with 4% isoflurane and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the brains were removed and embedded in paraffin. Coronal sections were cut into approximately  $2\ \mu\text{m}$  sections and stained with hematoxylin and eosin (H and E) or 0.1% cresyl violet for Nissl staining and prepared for subsequent microscopic mounting. Histopathological changes in the

brain were observed under an optical microscope (Leica Microsystems Ltd., Wetzlar, Germany). In each CA1 region of the hippocampus, the number of intact-appearing pyramidal cells showing a distinct nucleus and nucleus was counted of along a 1.35 mm transection ( $\times 50$  magnification) in light overview of microscopy. The number of pyramidal cells per mm in each group was expressed as the mean of the three coronal sections.

### Immunohistochemistry

For immunohistochemistry, brain sections were deparaffinized, rehydrated, and preincubated in 2% bovine serum albumin containing 0.3% Triton X-100 for 30 min. After washing in  $1 \times$  phosphate buffered saline, the sections were incubated with primary antibodies against the glial fibrillary acidic protein (GFAP; Abcam<sup>®</sup>, Cambridge, MA, USA), and neuronal nuclei (NeuN) (EMB Millipore, Billerica, MA, USA) at 4°C overnight. Following washing and incubation with the streptavidin-biotinylated secondary antibody (Ab) (Abcam<sup>®</sup>), the sections were visualized diaminobenzidine. The numerical density of GFAP-or NeuN-immunoreactive cells in a unit area was counted in the hippocampus CA1 region. The number of the cells per mm<sup>2</sup> in each rat was expressed as the mean of the three different sections.

### Fluoro-Jade B staining

To investigate the neuroprotective effect of mBHT on the pyramidal neuronal damage in the CA1 region of hippocampus in 2VO rats, Fluoro-Jade (F-J) B (a high affinity fluorescent marker for the localization of neuronal degeneration) staining was performed. In brief, brain sections were immersed in 1% sodium hydroxide in 80% alcohol and followed in 70% alcohol. The sections were then transferred to a 0.06% potassium permanganate, and then to a 0.0004% F-J B solution (Histochem, Jefferson, AR, USA). After washing in distilled water, the sections were placed on a slide warmer at 50°C, and then examined using a fluorescent microscope (Leica Microsystems Ltd.) with blue excitation light (450–490 nm) and a barrier filter.

### Immunofluorescence

In order to highlight the differences in the levels of activated nuclear factor-kappa B (NF- $\kappa$ B) between CA1 pyramidal neurons and astrocytes within this region of the hippocampus, double-labeling of GFAP or NeuN and NF- $\kappa$ B was carried out. Brain sections were double-labeled using the anti-NF- $\kappa$ B Ab (Cell Signaling Technology, Danvers, MA, USA) and also anti-GFAP or anti-NeuN primary Ab. An anti-alexa 488-conjugated (for label GFAP or NF- $\kappa$ B, green), and anti-alexa 568 (for label NeuN or NF- $\kappa$ B, red) secondary antibodies (Molecular Probes, Eugene, OR, USA), and 4,6-diamidino-2-phenylindole were used as a substitute of biotinylated secondary Ab.

All brain sections were observed under a fluorescence microscope (Leica Microsystems Ltd.).

### Western blot

On day 42, all rats were killed, and brain tissues were removed, and isolated the hippocampus. The hippocampus tissues were homogenized for 30 min at 4°C of radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% triton  $\times 100$ , 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], add 1 mM phenylmethylsulfonyl fluoride and proteintase inhibitor cocktail), and then lysed in SDS buffer. The protein concentration was determined using the Bradford's assay solution and the sample (20  $\mu$ g) was separated on SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in  $\times 1$  tris buffered saline (TBS). The membranes were incubated at 4°C overnight with primary antibodies against Bax (Cell Signaling Technology), Bcl-2 (Cell Signaling Technology), caspase-3 (Cell Signaling Technology) and  $\beta$ -actin Ab (Sigma-Aldrich Co. LLC., Sigma, CA, USA), respectively. After washing with  $\times 1$  TBS, the membranes were incubated with secondary antibodies, anti-rabbit immunoglobulin G (IgG) or anti-goat IgG (Santa Cruz Biotechnologies, Inc.) for 2 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence reagent (ECL, Bio-Rad Laboratories, Hercules, CA, USA), and apparent molecular weights (MWs) were assigned to the specific bands using prestained standards MWs (Bio-Rad Laboratories). Finally, bands were quantified by a computerized densitometer.

### Statistical analysis

The data were presented as mean  $\pm$  standard error of the mean value. Statistical analysis was carried out by One-way analysis of variance followed by Duncan's Multiple Range Test using the Software Graphpad Prism 5.0 (GraphPad Prism Inc., USA). Treatment mean differences with  $P < 0.05$  were considered statistically significant.

## RESULTS

### Effect of modified Bo-yang-Hwan-O-Tang on neuropathological changes of hippocampal CA1 neurons

The CA1 pyramidal neuronal cells in the hippocampal region may spontaneously regenerate following global cerebral ischemia.<sup>[15,16]</sup> Therefore, we investigated the effect of mBHT on neuronal depletion in the hippocampal CA1 region by chronic cerebral hypoperfusion in 2VO rats. In H and E staining, severe neuronal losses and morphological change were observed in the CA1 area of



2VO group [Figure 1a]. In 2VO group, dying neuronal cells in the CA1 showed shrunken cytoplasm and nucleic degeneration characterized by pyknotic and indistinct nuclei [Figure 1b], and the number of surviving neurons was significantly decreased [Figure 1c]. However, the administration of mBHT at doses of 250 and 500 mg/kg significantly increased the numbers of intact neuronal cells containing large, round, and transparent nuclei compared with that of 2VO group.

We next investigated the effect of mBHT on neuronal survival in the hippocampal CA1 region by Nissl and F-J B staining [Figure 2]. In Nissl staining, the losses of Nissl-positive viable neuronal cells with typical neuropathological change were observed in 2VO group. However, the administration of mBHT in 2VO group significantly increased the numbers of Nissl-positive neuronal cells in CA1 compared with that of 2VO group. In F-J B staining, F-J B positive-neuronal degeneration in the hippocampal CA1 region was observed in 2VO group while this appearance prevented strongly by the administration of mBHT at doses of 250 and 500 mg/kg. These results indicate that mBHT exerts neuroprotective effect on the ischemic damage of pyramidal neuronal cells in the hippocampal CA1 region in 2VO rats.

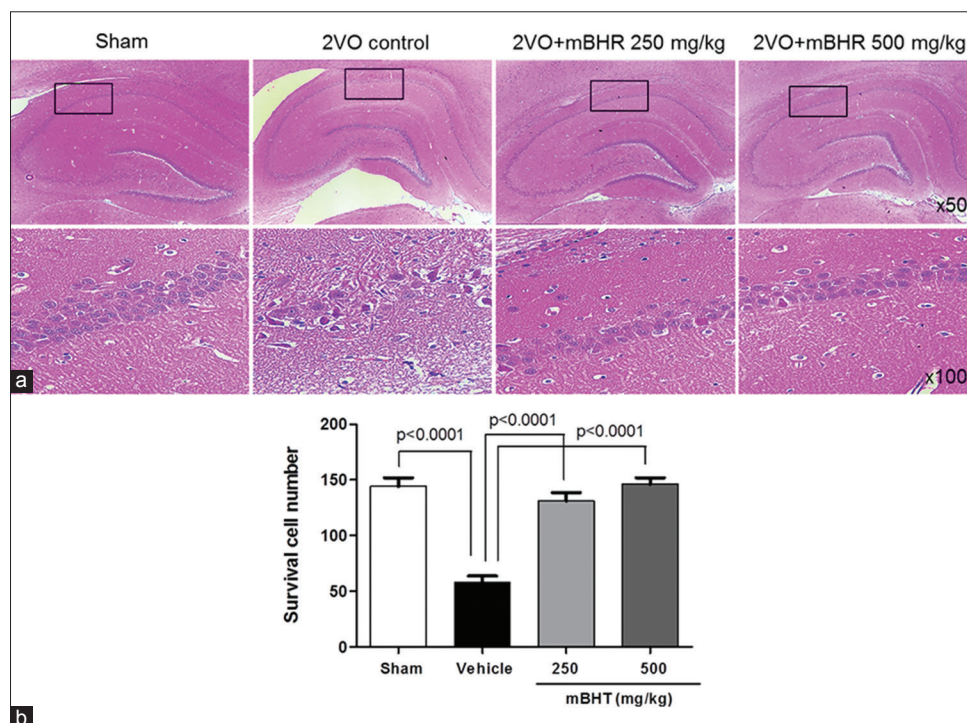
#### Effect of modified Bo-yang-Hwan-O-Tang on astrogliosis in hippocampal CA1 region

The hippocampal damage observed in 2VO and various acute ischemic models were evidenced by a massive

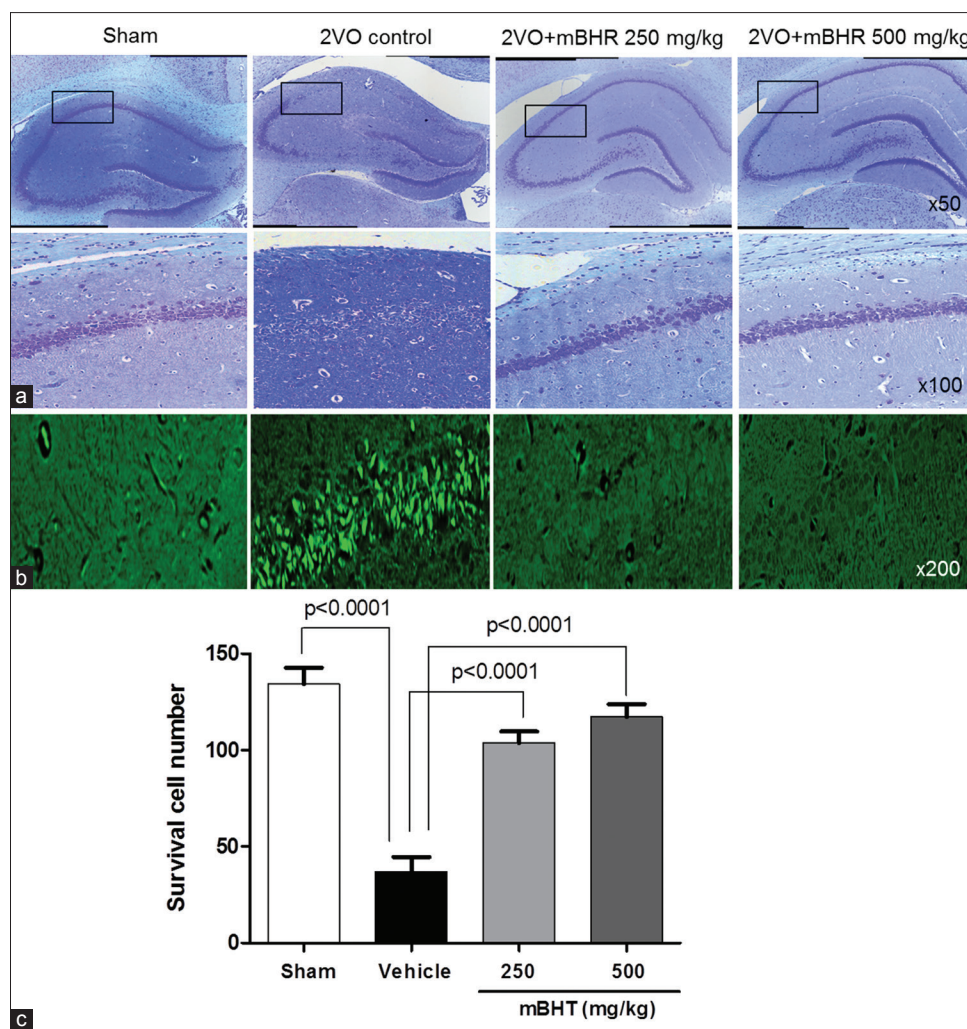
neuronal loss of the CA1 region that was accompanied by an intensive increase in GFAP labeling, indicating an astrogliosis process.<sup>[17]</sup> Therefore, we here investigated the effect of mBHT on astrogliosis process with the CA1 neuronal damage in 2VO rats. As shown in Figure 3, reactive astrocytic bodies were significantly increased with large GFAP-positive astroglial processes in the CA1 region of 2VO group. This astroglial change was inhibited by the administration of mBHT at doses of 250 and 500 mg/kg with a significant decrease of the number of GFAP-positive astrocytes [Figure 3c].

#### Effect of modified Bo-yang-Hwan-O-Tang on the distribution of active nuclear factor-kappa B in hippocampal CA1 region

First, to investigate the effect of mBHT on hippocampal damage accompanied by massive loss of neuronal cells and the astrogliosis in 2VO rats, double-immunofluorescence staining was performed using anti-NeuN and anti-GFAP antibodies. As shown in Figure 4, evident loss of NeuN-labeled viable neurons and the increase of GFAP-labeled astrocytes in the hippocampal CA1 region were observed in 2VO group. However, these neuronal loss and astrogliosis in 2VO rats were a little observed in mBHT-administrated group. Meanwhile, the activation of NF- $\kappa$ B protects hippocampal neurons against oxidative stress in cerebral ischemia and enhance memory and learning formation while its inhibition in glia might ameliorate the disease process.<sup>[18,19]</sup> Therefore, we investigated whether mBHT can regulate the NF- $\kappa$ B



**Figure 1:** Effect of modified Bo-yang-Hwan-O-Tang on the hippocampal CA1 neuronal damage in two-vessel occlusion rats. (a) hematoxylin and eosin staining; and (b) the number of surviving cells in the CA1 square box. All values are expressed as mean  $\pm$  standard error of the mean ( $n = 6$  per a group)



**Figure 2:** Effect of modified Bo-yang-Hwan-O-Tang on the hippocampal CA1 neuronal degeneration in two-vessel occlusion rats. (a) Nissl; (b) Fluoro-Jade stain and (c) the number of surviving cells in a square box. All values are expressed as mean  $\pm$  standard error of the mean ( $n = 6$  per a group)

activation in the hippocampal CA1 neurons or activated astrocytes using double immunofluorescence staining. As shown in Figure 4, the co-localization between GFAP-labeled astrocytes and NF- $\kappa$ B around the CA1 was observed in 2VO group, but NF- $\kappa$ B expression did not observed in NeuN-labeled neurons in the CA1. However, the administration of mBHT in 2VO rats inhibited the NF- $\kappa$ B co-localization with GFAP-labeled astrocytes. The results indicate that the treatment of mBHT in 2VO rats might prevent the reactive astrocytes-mediated neuroinflammation and neuronal apoptosis.

#### Effect of modified Bo-yang-Hwan-O-Tang on the expression of apoptotic proteins in hippocampal CA1 region

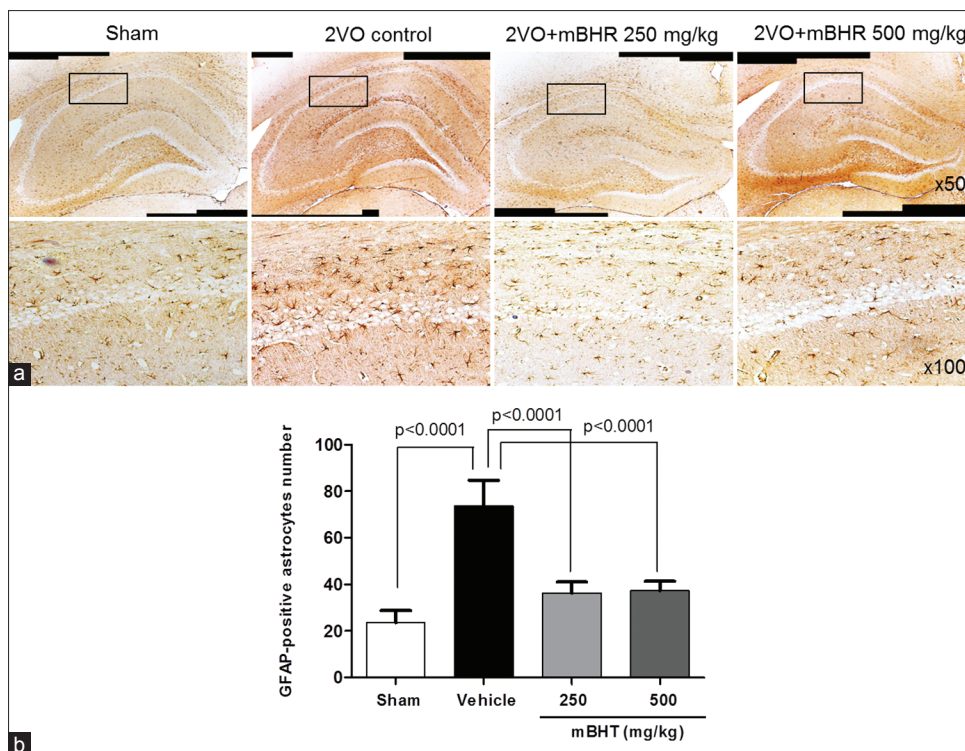
To investigate the effect of mBHT on the increase of apoptotic proteins following the hippocampal damage, the expression of bax, bcl-2, and caspase-3 was detected in the hippocampal CA1 region of 2VO rats by Western blot. In 2VO group, the ischemic activation was significantly

induced the increase of apoptotic proteins, bax and caspase-3, but significantly decrease of anti-apoptotic protein, bcl-2 [Figure 5a]. The treatment of mBHT at doses of 250 and 500 mg/kg prevented the downregulation of bcl-2 expression and the upregulation of bax expression, which resulted in decreasing bax/bcl-2 ratio in the hippocampus of 2VO rats [Figure 5b]. In addition, the treatment of mBHT significantly ameliorated the caspase-3 activation induced by permanent occlusion of bilateral common arteries. These results indicate that mBHT has a protective effect against 2VO-induced neuronal degeneration in the hippocampus [Figure 5].

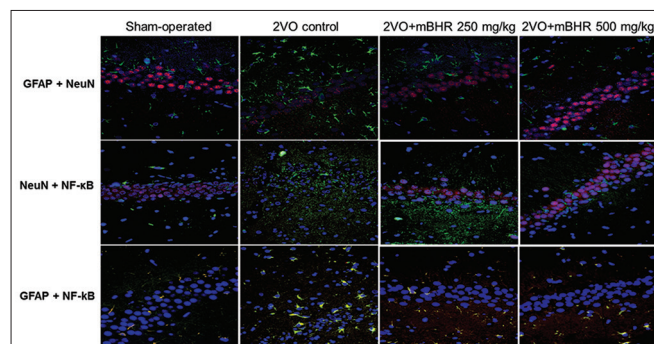
## DISCUSSION

The disease mechanism of VD is the central role that cerebral blood vessels play in brain health, not only for the delivery of oxygen and nutrients, but also for the trophic signaling that closely connects the well-being of neurons and glia to that of cerebrovascular cells.<sup>[20]</sup>





**Figure 3:** Effect of modified Bo-yang-Hwan-O-Tang on the astroglial activation in the hippocampal CA1 of two-vessel occlusion rats. (a) anti-gial fibrillary acidic protein (GFAP) antibody staining; and (b) the number of GFAP-positive cells in a square box. All values are expressed as mean  $\pm$  standard error of the mean ( $n = 6$  per a group)



**Figure 4:** Effect of modified Bo-yang-Hwan-O-Tang on the nuclear factor-kappa B (NF- $\kappa$ B) expression in hippocampal neurons and astrocytes of two-vessel occlusion rats. Brain tissues were stained with anti-neuronal nuclei, glial fibrillary acidic protein, and NF- $\kappa$ B antibodies, and observed by fluorescence microscope ( $\times 200$ )

VD is often coexisting with neurodegenerative diseases such as Alzheimer’s disease, and mixed vascular and neurodegenerative dementia has risen as the leading cause of age-related cognitive impairment. On the other hand, chronic cerebral hypoperfusion is considered as a factor that contributes memory dysfunction in VD through chronic disruption of cerebral blood flow resulting the neurological deficit and behavioral impairment.<sup>[5]</sup> Hence, the degree of chronic cerebral hypoperfusion has been suggested as a predictive biomarker of the gradual transition from a mild cognitive impairment to VD.<sup>[6]</sup> Actually, the patients of VD may not be able to stop the disease

progressing, but they may slow it down using medication to lower high blood pressure, acetylcholinesterase inhibitors, memantine, and cerebrolysin as a new drug and various therapies such as cognitive stimulation, reality orientation, validation and behavioral therapy.<sup>[21]</sup> Therefore, there are recently studying new possibility of traditional medicines as a treatment for behavioral and psychiatric symptoms in VD.<sup>[22]</sup>

Modified Bo-yang-Hwan-O-Tang is an improved herbal formula of BHT, which has been traditionally used to treat paralytic patients as well as for the treatment of stroke, senility, VD and heart damage by the addition of five herbs having neuroprotective and vasoprotective properties.<sup>[23,24]</sup> mBHT is newly developing as a natural drug consisting twelve herbs for the treatment of ischemic stroke. In this study, we prepared the standardized mBHT from the pharmaceutical company and established the condition for specifications and test procedures of drug according to the guideline of the Korea Food and Drug Administration.<sup>[24]</sup> mBHT is believed to promise a good therapeutic candidate for ischemic stroke with various efficacies such as thrombolysis, immune-modulation, anti-inflammation, anti-oxidation, neurovascular protection, and reducing the infarct volume, and edema and ameliorating pathophysiological symptoms with neurological deficits, and neuroinflammation in transient middle artery carotid

occlusion-induced ischemic rats.<sup>[10-14]</sup> In this study, we evaluated another therapeutic potential of mBHT on hippocampal neuronal death in chronic cerebral hypoperfusion-induced VD.

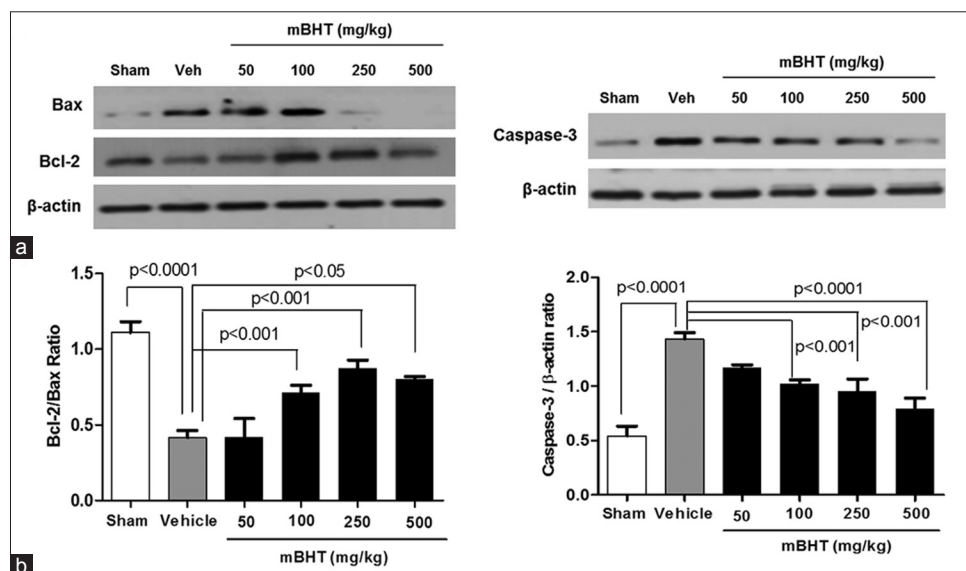
Permanent bilateral ligation of the common carotid arteries (2VO) in rats is a chronic cerebral hypoperfusion *in vivo* model that can induce the reduction of cerebral blood flow.<sup>[25]</sup> This model causes chronic impairments in working memory and primarily hippocampal damage with loss of CA1 neurons, axons, and activation of astrocytes, similar the effects observed in a human VD.<sup>[26]</sup> Therefore, 2VO rats are widely used for understanding the pathophysiology of chronic cerebrovascular diseases and to screen drugs with potential therapeutic value in VD. Our study was found that the administration of mBHT in 2VO rats markedly attenuated hippocampal neuronal loss/death induced by chronic cerebral hypoperfusion. This anti-apoptotic action of mBHT might be involved in the therapeutic effect in VD.

Delayed neuronal death follows chronic cerebral hypoperfusion in vulnerable region of the brain, especially in the hippocampus, which is highly sensitive to ischemic insults and plays an important role in learning and memory.<sup>[20]</sup> It is considered that the permanent 2VO rats show neurological impairment due to chronic cerebral hypoperfusion. In our study, the hippocampal damage observed in 2VO rats was evidenced by a massive neuronal loss in the hippocampal CA1 region that was accompanied by an intense increase in GFAP staining, indicating an astrogliosis. However, the administration of mBHT in 2VO rats effectively

prevented this neurodegeneration in hippocampal CA1 region, suggesting that mBHT has a neuroprotective effect against massive neuronal death following chronic cerebral hypoperfusion. In the other hand, it was known that permanent occlusion of bilateral common carotid arteries caused an early-emerging impairment of Morris water maze acquisition and late-emerging CA1 cell loss.<sup>[22]</sup> In a future study, it is necessary to explore the effect of mBHT on cognitive impairment by pyramidal neuronal loss in the hippocampus using Morris water maze test in 2VO rats.

Astrocytes are close morphological and functional association with neurons and importance of these glial cells in brain disorders has been strongly suggested.<sup>[27]</sup> After brain injury, astrocytes undergo morphological changes, extend their processes and increase synthesis of GFAP. GFAP is a marker for changes in reactive astrocytes during brain development/injury and form a dense web of their plasma membrane extensions that fills the empty space generated by the dead or dying neuronal cells as a process called astrogliosis.<sup>[28]</sup> In our study, chronic cerebral hypoperfusion in 2VO rats caused a significant GFAP increase in the hippocampus, but this decreased after the administration of mBHT. This result suggests that mBHT might be inhibited the chronic hypoperfusion-induced astrogliosis in VD.

Transcription factor, NF- $\kappa$ B has diverse functions in the central nervous system (CNS), depending on the cellular environment. In glutamatergic neurons of the CNS, constitutive NF- $\kappa$ B activity was found in the hippocampus granule cells and pyramidal CA1 neurons



**Figure 5:** Effects of modified Bo-yang-Hwan-O-Tang on the expression of Bax, Bcl-2 and caspase-3 in the hippocampus of two-vessel occlusion rats. (a) Western blot; and (b) the densitometric analysis of three independent experiments. All values are expressed as mean  $\pm$  standard error of the mean

and the cerebral cortex.<sup>[29]</sup> Hippocampal region-specific regulation of NF- $\kappa$ B may contribute to learning and memory formation through the consolidation-associated synaptic plasticity. However, we here did not find the co-localization between NeuN-labeled neurons and NF- $\kappa$ B in 2VO rats. On the other hand, in glia, it is inducible and regulates the inflammatory process, and mainly co-localize with GFAP in processing the later stage of neuropathic pain.<sup>[30,31]</sup> Hence, blockade of the NF- $\kappa$ B activation in the hippocampal astrocytes might attenuate nociceptive responses following neuronal damage.<sup>[32]</sup> Our study, the number of GFAP-labeled astrocytes in the hippocampus in 2VO rats highly induced. Furthermore, the administration of mBHT in 2VO rats decreased in GFAP-labeled activated astrocytes. This result suggests that mBHT produces anti-apoptosis effect through the inhibition of NF- $\kappa$ B activation by reactive astrocytes.

It has been widely accepted that chronic cerebral hypoperfusion induces oxidative stress damage and brain energy failure in neuronal cells that is related to cognitive impairment. Oxidative injury leads to release apoptosis-inducing factors and consequent activation of the cascade of enzymes and ultimately result in neuronal death by necrosis or apoptosis.<sup>[33]</sup> The bcl-2 and bcl-x<sub>L</sub> proteins suppress programmed cell death induced by various stimuli, whereas bax promotes cell apoptosis.<sup>[34]</sup> Bax, bcl-2 and bcl-x proteins are all expressed in the nerve systems of developing and neonatal rats, suggesting that these proteins regulate neuronal cell death during development. However, bax overexpression in sympathetic neurons induces cell death, which can be blocked by co-expression of bcl-2 or a caspase inhibitor. Increased ratio of bax/bcl-2 up-regulates caspase-3 which is an apoptosis co-ordination enzyme and increases neuronal apoptosis and functional impairment following cerebral ischemia/reperfusion injury in rats.<sup>[35]</sup> In our study, the treatment of mBHT prevented the downregulation of bcl-2 expression and upregulation of bax expression, which resulted in decreasing bax/bcl-2 ratio in the hippocampus of 2VO rats. In addition, mBHT effectively alleviated the activation of caspase-3 induced by chronic cerebral hypoperfusion. These results suggest that mBHT exhibits therapeutic potential for VD, which is most likely related, at least in part, to its antioxidation and anti-apoptotic actions.

## CONCLUSION

Our study first demonstrated that mBHT, a polyherbal medicine for ischemic stroke, prevents chronic cerebral hypoperfusion-induced hippocampal neurodegeneration through inhibiting the neuronal death and astrogliosis in 2VO rats. These results suggest that mBHT has a good

therapeutic potential for the treatment of VD by chronic cerebral hypoperfusion.

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