Brahmi Extract Attenuated Spatial Learning and Memory Impairment and Cell Death of Rat Hippocampal CA1 Neurons after the 2-VO Induced Chronic Cerebral Hypoperfusion

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ABSTRACT

Background: Vascular dementia, a learning and memory afflicted disease, is caused by chronic cerebral hypoperfusion which leads to neuronal injury and death. Brahmi extract is used in Indian Ayurvedic medicine to enhance learning and memory and to promote brain functions.

Objective: To examine the therapeutic potential of Brahmi extract as an alternative treatment for vascular dementia in the rat model of chronic cerebral hypoperfusion.

Methods: The chronic cerebral hypoperfusion was induced by permanent bilateral common carotid artery occlusion (2-VO), causing vascular dementia. After the occlusion, Brahmi extract was given for 2 months, and the spatial learning and memory tests were then performed after 2 months of drug administration. At the end, all animals were sacrificed, and the brains were removed for histological examination and counting of the hippocampal CA1 neurons.

Results: The 2-VO induced cerebral hypoperfusion reduced the hippocampal CA1 neuron count and impaired the spatial learning and memory. Brahmi extract, on the other hand, could attenuate the memory deficit and neuronal reduction.

Conclusion: Brahmi extract attenuated the deficit induced by the permanent bilateral common carotid artery occlusion, suggesting the neuroprotective effects of Brahmi extract against the cerebral hypoperfusion.

Keywords: Cerebral hypoperfusion, Brahmi, common carotid artery ligation, 2-VO, Hippocampus, CA1

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INTRODUCTION

Vascular dementia or vascular cognitive impairment is the second most common form of dementia after Alzheimer's disease.[1,2] It is characterized by the sudden and/or progressive decline in cognitive and memory functions, which is induced by an insufficient blood supply to the brain.[3,4] The risk factors that contribute to the dementia are advanced age, genetic factors, and vascular risk factors such as hypertension, high blood cholesterol, and arteriosclerosis.[5-7] These risk factors lead to the moderated cerebral hypoperfusion, hemorrhagic or ischemic brain injuries both in cortical and subcortical brain regions.[6] Chronic cerebral hypoperfusion is the sustained reduction of cerebral blood flow which leads to the production of reactive oxygen species and the reduction of glucose and oxygen, the essential substances for the brain activities.[8] Previous evidence had shown the correlation between the memory deficit and decline in the cerebral blood flow in vascular dementia.[9,10] The hippocampus, the important brain area for spatial learning and memory processes,[11,12] was highly vulnerable to the cerebral hypoperfusion.[13,14] In addition, damage to the CA1 subregion of the hippocampus led to the decline in learning and memory.[15,16] In this experiment, the chronic cerebral hypoperfusion was induced in rats using the permanent bilateral common carotid artery occlusion or

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modified 2-VO model, subsequently causing the learning and memory impairment.[17]

Brahmi (BM) is an aquatic and creeping plant found in many Asian countries including Thailand.[18] It is widely used in Indian traditional system of Ayurvedic medicine to enhance learning and memory and to promote the brain functions.[19,20] Its scientific name is *Bacopa monnieri Wettst.* The active ingredients in Brahmi are saponins which can be subdivided into two groups, jujubogenin glycoside and pseudojujubogenin glycoside.[18] The administration of Brahmi extract in male rats with the doses of 20, 40 and 80 mg/kg for at least 2 weeks increased learning and memory capabilities.[21] Moreover, Brahmi extract could prevent the neuronal damage after the exposure to the varieties of chemicals.[22-24] For example, the treatment of Brahmi extract significantly reduced the learning impairment from the focal cerebral ischemia induced by transient internal carotid artery occlusion in stroke models, and this neuroprotective effect was likely due to the antioxidant activity of Brahmi extract.[25]

Therefore, several studies in neuronal cell cultures, animal models, and clinical studies have shown the strong evidences and suggested that Brahmi extract could prevent neuronal damage from brain insults, and promote normal learning and memory both in humans and animals.[21,23,26] However, there is no known study focusing on the protective effect of Brahmi extract against the damage of the hippocampal CA1 subregion and memory deficit induced by chronic cerebral hypoperfusion. Therefore, the aim of this experiment was to determine the possibility of using Brahmi extract in the prevention of the impairment caused by the 2-VO induced chronic cerebral hypoperfusion.

MATERIALS AND METHODS

Animals

Fifty five adult male Wistar rats, 4 weeks old and weighing between 180-210 grams, were used in total. They were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. Rats were maintained in the 12 hour light/dark cycle and were housed individually with ad labium access to food and water. All experiments were approved by the Animal Ethics Committee, Faculty of Medicine Siriraj Hospital, Mahidol University.

Preparation of Brahmi extract

The ethanolic extract of Brahmi plant was processed and provided by the Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok. The extraction processes followed method 4 which was done in a previous study.[27] In brief, the plants were dried and then cut into small pieces. The dried plant material was soaked in water for 24 hours and macerated with 95% ethanol for the next 3 days at room temperature. The resulting extract was filtered through filter papers. The filtrated residue was extracted again twice with the same procedure. The obtained filtrates were combined, and evaporated under reduced pressure. The concentrated extract was filtered through filter papers, and the final filtrate was dried using the spray-dried technique. The dry powder of Brahmi extract was stored in a desiccator and protected from light.

Experimental design

All rats were randomly allocated to one of five animal groups. In summary, the first group was a non-ischemic group which received sham operations, and was orally fed with 1 ml of vehicle, 0.9% normal saline. The rest were ischemic groups that received bilateral common carotid artery occlusion with the administration of different tested compounds for eight weeks. The second group was orally fed with 1 ml of 0.9% normal saline as a negative control. The third, fourth and fifth groups were orally fed with Brahmi extract solution with the concentrations of 120, 160 and 240 mg/kg of body weight, respectively. Different amounts of Brahmi extract were dissolved in 1 ml of 0.9% normal saline to make the final concentrations of 120, 160, and 240 mg/kg. Each group daily received 1 ml of either 0.9% normal saline or the different concentrations of Brahmi extract by intragastric gavage for eight weeks after the administration of permanent bilateral common carotid artery occlusion. After eight weeks of the compound ingestion, all animals were trained with Morris water maze (MWM) task to test their spatial learning and memory. After the completion of behavioral training, all animals were euthanized and the brains were removed for histological examination.

Surgical procedure

The modified 2-VO model in which bilateral common carotid arteries were permanently blocked has been described in Cechetti et al., 2010.[17] The surgical operation was performed after one week of the habituation period. Animals were anesthetized by the intramuscular injections of a ketamine (60 mg/kg) and xylazine (0.6 mg/kg) mixture. After the animals were unconscious, a small incision was made at the midline of the neck region. The right common carotid artery was identified, and permanently ligated with a silk thread. The skin and soft tissues were sutured. Two days later, the left common carotid artery received the same operation. In the sham-operated control, the animals received the surgical operation without the common carotid artery ligation. The animals were returned to their home cage after they were fully awake.

Behavioral testing

The Morris Water Maze (MWM) is the behavioral test for the spatial learning and memory. The small circular pool was 200 centimeters in diameter and 50 centimeters deep. The pool was filled with water to 25 centimeters in depth, and was divided into 4 quadrants. The glass escape platform was placed at the center of a quadrant (target quadrant). The visual cues such as a colored triangle shape were placed around the room in plain sight of the animals. During training, an animal was randomly placed into the water at a quadrant of the pool. Due to its instinctive behavior, the animals would try to escape from the water. Each time, the animals were allowed to swim for 120 seconds. If they could not find the platform, they were

guided and left on the platform for 15 seconds. After the training, they would learn to use the visual cues to guide them to the escape platform as quickly as possible. The images of animals performing the experiments were recorded by a video camera.

Morris water maze protocol

The MWM task chronologically consisted of three training trials;[28] the visible platform, the acquisition, and the probe trials. All rats received the same training trials 4 times per day.

For the visible platform trial, this trial was performed on the first training day. The escape platform appeared two centimeters over the water surface, so the animals could clearly see the platform. The sensorimotor performances and motivation to escape from the water maze of the animals was analyzed using the time spent to find the visible platform and expressed as escape latency.

For the acquisition trial, this trial was consecutively performed for 7 days after the visible platform trial. The platform was submerged two centimeters under the water surface. The animals now had to learn to use the visual cues to guide them to the platform. The spatial learning and memory was recorded and analyzed by using the time spent to find the platform and also expressed as escape latency.

For the probe trial, this trial was performed on the next day after the completion of acquisition trials. The platform was removed. The animal, in which its memory was unimpaired, normally would spend more time in the target quadrant than the other quadrants. The memory retention, the animal's ability to maintain the previously acquired memory, was analyzed using the time spent in the target quadrant and expressed as mean retention time.

Histological examination for the hippocampal CA1 neuron count

After the completion of behavioral training, all animals were sacrificed with an overdose of the ketamine and xylazine mixture, and the brains were removed. The paraffin sections were processed and cut as 10 µm thickness of hippocampal slices. In each brain, a series of 3 sections spaced at an interval of 200 µm was stained with 0.1% cresyl violet to examine neuronal damage. The hippocampus was viewed under a light microscope (Carl Zeiss Axio Imager M2, Germany), and photographs of the hippocampal CA1 subregion were taken. Then, three representative images of the CA1 subregion were cropped into a 0.1 mm^2 square (345 x 300 μm^2). The numbers of hippocampal CA1 neurons in all representative images were manually counted using the free UTHSCSA Image Tool 3.0 program (University of Texas Health Science Center at San Antonio). The detailed procedures have been described in Koomhin et al., 2012. [29]

Statistical analysis

The data were analyzed using SPSS 16.0. All results were expressed as mean ± SEM. The mean escape latency was analyzed using the repeated-measure ANOVA and followed by Fisher's least significant difference post hoc test. The other data were analyzed using one-way ANOVA and followed by Fisher's least significant difference post hoc test. *P* values of 0.05 or less were considered as significant.

RESULTS

1) Effects of Brahmi extract on sensorimotor performance and motivation

The visual performance, motor performance, and motivation of the animals were determined in the visible trial in which the platform was made visible. The results showed that the mean escape latencies were not significantly different among all groups [F (4, 54) = 0.651, *P* = 0.629] (Fig 1), indicating that the sensorimotor performance and motivation were not affected by either the Brahmi extract administration or 2-VO induced chronic cerebral hypoperfusion.

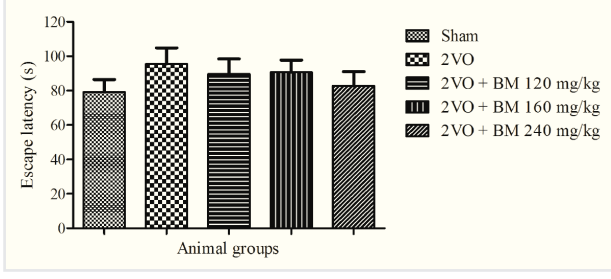

Fig 1. Effects of the 2-VO induced chronic cerebral hypoperfusion and Brahmi extract administration for two months, indicating the mean escape latencies in the visible trial of rats orally fed with either vehicle (Sham and 2VO groups) and varying doses of Brahmi extract (2VO+BM 120 mg/kg, 2VO+BM 160 mg/kg, and 2VO+BM 240 mg/kg of body weight), (n = 11 / group). Data are shown as mean ± SEM.

2) Effects of Brahmi extract on spatial learning and memory

Rats were tested for their spatial learning and memory performances in the acquisition trial. The 2-VO animals demonstrated significantly higher escape latency than the sham animals [F (1, 50) = 7.475, *P* = 0.000], indicating the impairment of spatial learning and memory after the 2-VO induced cerebral hypoperfusion (Fig 2). All doses of Brahmi extract could significantly decrease the mean escape latencies as compared to the 2-VO group [F (1, 50) = 7.475, $P \leq 0.002$]. The result indicated that Brahmi extract could attenuate the impairment of spatial learning and memory induced by chronic cerebral hypoperfusion.

In addition, memory retention was assessed by observing the time spent in the target quadrant (mean retention time) during the probe trial (Fig 3). The 2-VO animals had significantly lower retention time than the sham animals [F (4, 54) = 3.226, P = 0.002], indicating the deficit in the memory retention after chronic cerebral hypoperfusion. The administration of Brahmi extract 120 mg/kg in 2-VO mice could significantly prevent the deficit [F (4, 54) = 3.226, *P* = 0.005].

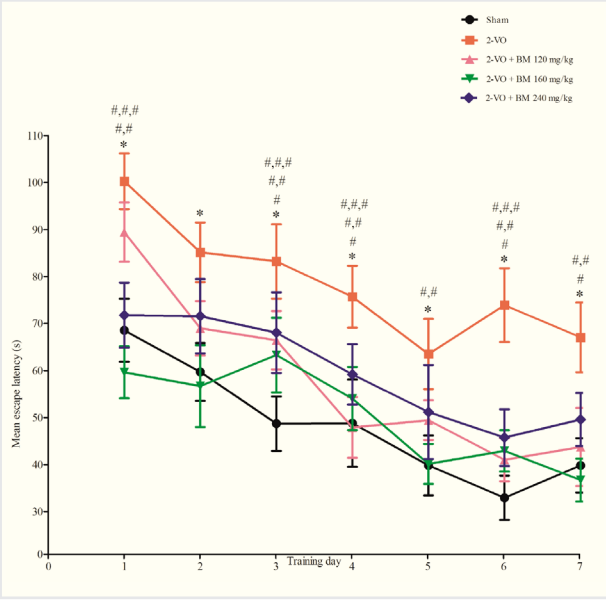

Fig 2. The mean escape latency in the acquisition trials. Data are shown as mean ± SEM (n = 11/group). "*" indicates significance at P < 0.000 of 2-VO group versus sham group. "#" indicates significance at P ≤ 0.002 of 120 mg/kg Brahmi group versus 2-VO group."#,#" indicates significance at P ≤ 0.002 of 160 mg/kg Brahmi group versus 2-VO group. "#,#,#" indicates significance at P ≤ 0.002 of 240 mg/kg Brahmi group versus 2-VO group.

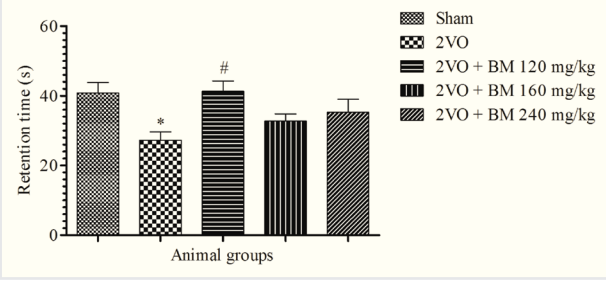

Fig 3. The time spent in the target quadrant (retention time) in the probe trial. Data are shown as mean ± SEM (n = 11/group). "*" indicates significance at P = 0.002 of 2-VO group versus sham group. "#" indicates significance at P = 0.005 of each Brahmi treated group versus 2-VO group.

3) Effects of Brahmi extract on the number of hippocampal CA1 neurons

Moreover, the effect of Brahmi extract on the number of survival neurons in the CA1 subregion of the hippocampus was examined (Fig 4). The survival neurons were identified by their large nuclei and prominent nucleoli. The numbers of survival neurons per total area (0.3 mm^2) were counted. The 2-VO animals showed the significant reduction of survival CA1 neurons from the control level [F (4, 14) = 5.684, P= 0.005] (Fig 5). However, all doses of Brahmi treated animals showed significantly higher numbers of hippocampal CA1 neurons than the 2-VO animals [F (4, 14) = 5.684, P = 0.006; F (4, 14) = 5.684, P = 0.011; F (4, 14) = 5.684, P = 0.001 for BM 120, 160 and 240 mg/kg, respectively], suggesting that the 2-VO which induced the reduction of survival neurons was effectively mitigated by Brahmi extract administration. These results indicated that the 2-VO induced chronic cerebral hypoperfusion decreased the number of survival hippocampal CA1 neurons, and Brahmi extract could attenuate this deleterious effect, suggesting the neuroprotective effect of Brahmi extract on chronic cerebral hypoperfusion.

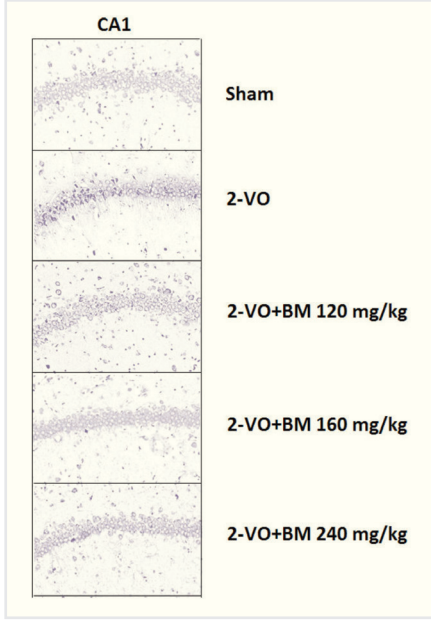

Fig 4. The 10 um thick slides of each representative section in the CA1 area of the hippocampus. The nissl bodies of the pyramidal cells were stained with 0.1% cresyl violet. The pictures from the top to bottom panels represent the different animal groups (Sham, 2-VO, 2-VO+BM 120 mg/kg, 2-VO+BM 160 mg/kg, 2-VO+BM 240 mg/kg, respectively).

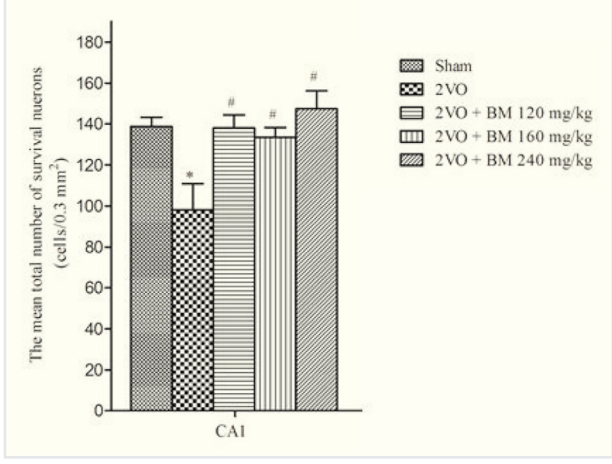

Fig 5. Mean number of survival hippocampal CA1 neurons (n = 3/group). The results are shown as mean ± SEM. "*" indicates significance at P ≤ 0.05 of 2-VO group versus sham group. "#" indicates significance at P ≤ 0.05 of each Brahmi treated group versus 2-VO group.

DISCUSSION

The sustained reduction of cerebral blood flow in aged people is a pathological mechanism which leads to vascular dementia, a neurodegenerative disease that can cause severe cognitive impairment.[6] In this experiment, the global reduction of cerebral blood flow was induced by the modified 2-VO procedure.[17] Right after the occlusion, the blood flow of the hippocampus was reduced substantially to about 55% of the normal level, then the blood flow gradually increased and returned to the normal level after 2 months.[30] Therefore, the 2-VO model could institute the chronic cerebral hypoperfusion condition.[8] The chronic cerebral hypoperfusion could activate several pathological mechanisms such as glutamate-mediated excitotoxicity, oxidative stress and inflammation, leading to neuronal damage and death.[31] Glutamate-mediated excitotoxicity was significantly related to the excess of intracellular calcium which in turn triggered the free radical formation within cytoplasm, and oxidative stress production.[32,33] The oxidative stress is defined as an imbalance between oxidants and antioxidants.[34] In chronic cerebral hypoperfusion, oxidants were over expressed more than the antioxidants.[35] Oxidant/anti-oxidant systems were the defense mechanisms of the body to reduce free radical formation and enzymes critical to this process included catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSH).[36] When the oxidative stress was increased, the free radicals such as nitric oxide (NO), superoxide (O_2^-) and peroxynitrite (ONOO –) were dramatically produced, causing neuronal damage.[37-40] Moreover, oxidative stress also activated inflammatory responses. The pro-inflammatory cytokines and chemokines which were released from injured and dead neurons activated microglia and astrocytes to release cytokines such as IL-6 and TNF-α.[41] These pro-inflammatory cytokines further led to the neuronal injury and death.[42] In this study, the deleterious effect of oxidative stress was shown in the 2-VO induced cerebral hypoperfusion animals in which the 2-VO rats significantly demonstrated the reduction of hippocampal CA1 neurons, and the impairment of spatial learning and memory.

Previous studies reported the neuroprotective effect of Brahmi extract against various brain insults.[22-24] In the present study, after the daily administration of varying doses of Brahmi extract, all Brahmi treated animals showed significantly lower mean escape latency than the 2-VO animals in the acquisition trial, and 120 mg/kg Brahmi treated animals exhibited significantly higher retention time than the 2-VO animals in the probe trial. The results indicated that the 2-VO induced spatial learning and memory deficits were mitigated by Brahmi extract, suggesting the protective effects of Brahmi extract against the chronic cerebral hypoperfusion. Although only the 120 mg/kg Brahmi extract demonstrated the significant result in the improvement of memory retention, the other Brahmi extract groups still showed the better result than the 2-VO group, still supporting the neuroprotective effect of Brahmi extract. However, the preferentially positive effect of the lower dose of Brahmi extract on the memory retention will be investigated in a further study. The mechanisms underlying this neuroprotective effect of Brahmi extract in spatial learning and memory were investigated by examining the amount of survival hippocampal CA1 neurons remaining after the chronic cerebral hypoperfusion. The data indicated that all doses of Brahmi extract significantly prevented the reduction of neurons in the CA1 subregion. It has been well recognized that the successful spatial learning of the MWM task highly depended on the activities of hippocampal neurons.[43] The hippocampus plays a critical role in the spatial learning and memory.[11,12] The CA1 neurons provided the major efferent of the hippocampus, and the lesion of CA1 led to the deficit in the learning and memory.[15,16] The 2-VO induced cerebral hypoperfusion caused the decrease in the numbers of CA1 neurons, perhaps, because the CA1 neurons had a high sensitivity to the brain ischemia.[44] In accord with other studies, the 2-VO animals in this study also demonstrated the deficit in spatial learning and memory.[8]

Brahmi extract could attenuate the decrease of hippocampal CA1 neurons and memory impairment after the 2-VO procedure. The possible neuroprotective mechanisms of Brahmi extract on chronic cerebral hypoperfusion were antioxidant, anti-inflammation and acetylcholinesterase (AChE) inhibitor.[22,45,46] Brahmi administration increased the levels of antioxidative enzymes such as catalase, SOD and GSH, which might have helped to decrease the excess of free radicals, and to protect the brain from oxidative stress induced by chronic cerebral hypoperfusion.[25] The detoxification mechanisms were done through the activities of many enzymes. Firstly, SOD converted the superoxide to hydrogen peroxide (H_2O_2), and the H_2O_2 was then removed via the catalytic activities of catalase and GSH.[47] Moreover, rats with carrageenan-induced paw inflammation showed the increase in TNF-α and IL-6 protein levels. After the administration of Brahmi extract, these protein levels were decreased as compared to the untreated group. The results demonstrated that Brahmi extract had an anti-inflammatory activity by decreasing pro-inflammatory cytokines.[45] Another hypothesis of memory impairment induced by chronic cerebral hypoperfusion is the central cholinergic hypofunction. The basal forebrain cholinergic neurons innervated the hippocampus via the fimbria and dorsal fornix.[48-50] These nerve terminals released acetylcholine (ACh), a neurotransmitter involved in learning and memory.[51] The previous studies found that rats with the 2-VO procedure showed the decrease in the number of cholinergic nerve terminals and ACh levels in the hippocampal formation.[48] The effect of Brahmi extract on cholinergic function was investigated. The evidence showed that Brahmi extract decreased AChE activity leading to the increase in ACh levels within the hippocampus, improving the impaired memory in the animal model.[46]

CONCLUSION

Brahmi extract showed neuroprotective effects against chronic cerebral hypoperfusion induced neuronal

damage in the modified 2-VO rat model. The possible protective mechanisms might be mediated by its antioxidative, AChE inhibitor, and anti-inflammatory activities.

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