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The Effects of *Dracocephalum multicaule* on the Cognitive Impairment and Hippocampal Neurodegeneration Induced by Chronic Cerebral Hypoperfusion

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Abstract

Introduction: Cognitive decline induced by chronic cerebral hypoperfusion (CCH) is the most common problem during ageing. Most of the synthetic drugs used for cognitive impairment have undesirable side effects in patients. Therefore, in the current research, the effects of *Dracocephalum multicaule* on cognitive impairment and hippocampal neurodegeneration induced by CCH were investigated.

Methods: CCH or sham surgical procedure was carried out in male Wistar rats. After three weeks, the animals were randomly assigned to *D. multicaule* extract or vehicle administration for 15 days. CCH induction was carried out by the bilateral occlusion of the common carotids. Morris water maze (MWM) and Nissl staining were used to evaluate cognitive function and hippocampal neuron density in rats, respectively.

Results: *Dracocephalum multicaule* administration increased the time spent in the target quadrant in CCH rats during the MWM test ($P<0.05$). *D. multicaule* improved hippocampal neuronal cell density in CCH animals ($P<0.05$).

Conclusion: *Dracocephalum multicaule* can improve spatial memory and hippocampal neuronal injury caused by CCH.

Keywords: Common carotid occlusion, *Dracocephalum multicaule*, Hippocampus, Cognition

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Introduction

Physiological functions of the organs decrease during aging as an inevitable process. Additionally, aging causes an irreversible decline in the cognitive process. Studies have shown a strong connection between aging and many chronic diseases in humans such as Parkinson's disease, cardiovascular disease, and Alzheimer's disease (AD). The risk of these diseases increases in the elderly.[1]

A chronic decrease in the cerebral blood supply is the main mechanism that can cause cognitive impairment. Bilateral obstruction of common carotids, known as 2-vessel occlusion (2VO), has been used for induction of chronic cerebral hypoperfusion (CCH) in rats.[2] 2VO is a suitable model for studying the interactions between brain hypoperfusion, neuronal damage, and memory deficits in AD and other neurologic diseases.[1]

Sudden interruption in blood flow to the brain tissues can lead to ischemia, whereas a mild and permanent decline in cerebral blood flow can affect cognitive function and cause neurodegenerative diseases.[3] CCH can also reduce dendritic branching and synapse formation.[4] CCH causes 60% reduction in cerebral blood flow in the hippocampus.[2] The hippocampus is the main region of CNS that is involved in learning and memory.

Dracocephalum multicaule is an annual species of the Lamiaceae family whose essential oil is used in traditional medicine, as well as food and health industries. *Dracocephalum* species (Lamiaceae) are aromatic plants that are found in northern China, central Asia, and eastern and central Europe.[5] *D. multicaule* has been used as one of the traditional remedies for cardiovascular diseases, including angina, hypertension, and atherosclerosis.[6-8]

This plant has many effects on the CNS as pretreatment with *D. multicaule* can attenuate neuronal damage after acute cerebral ischemia in rats.[9] This plant can reduce oxidative stress and neuro-inflammation in streptozocin-induced AD rat models.[10,11] The main components of *D. multicaule* include luteolin, chlorogenic acid, oleanolic acid, caffeic acid, ferulic acid, acacetin, quercetin, kaempferol, rosmarinic acid, and apigenin.[12] Jeon et al revealed that oleanolic acid can improve scopolamine-induced cognitive decline.[13] Finally, numerous studies have reported the cholinesterase inhibitory effect of the species Dracocephalum.[10,14]

In this study, we examined the effect of *D. multicaule* extract on the cognitive impairment caused by CCH in rats. Furthermore, we clarified its effects on neurodegeneration induced by CCH in the hippocampus (CA1 region) of rats.

Materials and Methods

Male Wistar rats (weighing 260 to 280 g) were obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). Rats were maintained under standard conditions. All experiments using animals were approved by the Ethics Committee for Animal Experimentation of the university. In this research, 40 rats were divided into four groups: (1) control, the sham animals that received vehicle, (2) *D. multicaule*, the sham animals that were treated with *D. multicaule* extract, (3) CCH, the CCH animals that received vehicle, and (4) CCH + *D. multicaule*, the CCH animals that were treated with *D. multicaule* extract.

CCH Induced by Bilateral Common Carotid Occlusion (2VO)

CCH induction was carried out using the 2VO (bilateral common carotid occlusion) surgery. The surgical method we used was similar to the one explained by Ritchie.[15] First, the animals were anesthetized with xylazine (10 mg/kg) and ketamine (90 mg/kg). A ventral midline incision on the neck was made. Then, common carotid arteries were detached from the surrounding muscle and vagus nerve and permanently ligated with a 5-0 silk suture. The survival rate after the surgery was 95%. Rectal temperature was maintained using a heating pad during the surgical procedure.

Dracocephalum multicaule Administration

Aerial parts of *D. multicaule* were purchased from Rahnamakesht Co., Isfahan, Iran (voucher number: 45869). Plant material was dried in the shade and ground to powder. Hydroalcoholic extract was obtained by percolation method using 70% ethanol with a ratio of 10:1 (10 mL of solvent/1g of the plant). All the treatments were made at 9 to 11 AM. Animals received the vehicle or *D. multicaule* extract by gavages at 10 mg/kg for 15 consecutive days. *D. multicaule* administration started 21 days after sham or CCH surgery.

Morris Water Maze Test

Twenty-four hours after the last dose administration of *D. multicaule* or vehicle, Morris water maze (MWM) test was started. This apparatus consisted of a large round pool, containing water at 25 °C. The diameter of the pool was 1.8 m. A Plexiglas platform (diameter of 10 cm) was placed in the center of the north-east quarter of the maze and 2 cm under the water surface. An automatic filling and draining system was used for filling and draining the pool. A video camera, connected to a computer, was fixed above the pool. The walls of the room were covered with some visual cues. Immediately prior to behavior testing, the rats were placed in the pool for 10 minutes for adaptation. Each animal was put in the water from one of the north, south, east, or west quarters to find the hidden platform. If the animal could not find the platform within one minute, it was directed by hand to find the platform and after that, it was allowed to stop on it for 45 seconds. Animals were trained in a water maze for four days. The memory function was evaluated 24 hours after the last training trials. The probe test lasted for 60 seconds, in which after removing the platform, the animal was placed in the water from the opposite side of the platform and allowed to find the platform. During the training trials, swimming speed, latency time, and traveled distance to find the platform were measured. During the probe trials, percentage of the time spent in the north-east quarter (target quadrant) was calculated.

Nissl Staining

After probe trials, animals were perfused with sodium chloride (0.9%) and then paraformaldehyde (4%) in 0.1 M phosphate. After decapitation and removing the brain from the skull, the brain was kept in paraformaldehyde solution. Paraffin embedding was carried out after 48 hours. Sections (7 μm) containing the hippocampus were prepared (bregma 3.6 to 4 mm). For Nissl staining, of 6 sections, one was randomly selected. Staining was carried out using 1% cresyl violet acetate (sigma) solution. Light microscope and OLYSIA bio-report image analysis software (Olympus, New South Wales, Australia) were used for estimation of the total number of pyramidal neurons in CA1 area (400x magnification). Cells with distinct nucleus were considered living cells, and dark and shrunk cells were considered dead cells.

Data Analysis

The data were represented as mean ± SD. After checking the normality of the data, two-way repeated measures analysis of variance was used for analyzing the data of the training trials (traveled distance, swimming speed, and latency time), and one-way ANOVA was used for analyzing the rest of the data. Tukey's post hoc test was

used for between-group comparisons. The significance level was $P<0.05$. SPSS was used for data analysis.

Results

Morris Water Maze

Training Trials

During the four days of training trials in the MWM, there was no significant difference between groups in the traveled distance, swimming speed, and latency time. However, a significant within-group difference was revealed in the distance traveled during trial days (df1, 3 F = 11.58, $P<0.001$) (Figure 1). Two-way repeated measures analysis of variance showed significant within-group difference in swimming speed (df1, 3 F = 4.46, $P<0.01$) and latency time (df1, 3 F = 13.01, $P<0.001$) (Table 1). Analyzing the data of the training trials revealed that rats learned the task.

Probe Trials

Percentage of the time spent in the target quarter significantly decreased in CCH animals compared to the controls ($P<0.01$). *D. multicaule* increased the time spent in the target quadrant in CCH animals. CCH + D. multicaule animals spent significantly more time in the target quadrant compared to the CCH group ($P<0.05$) (Figure 2).

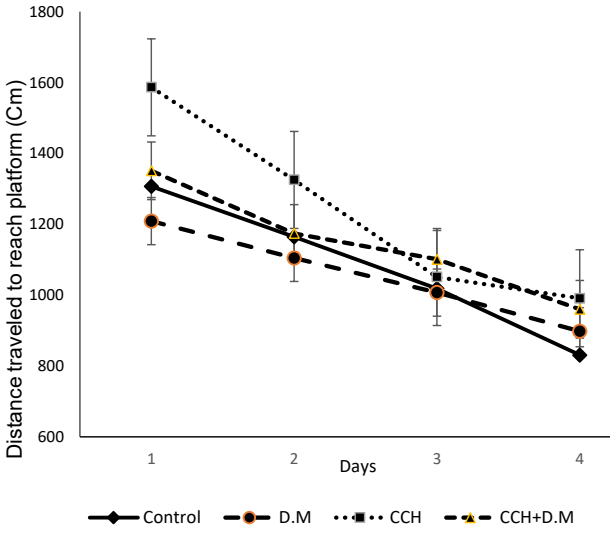

Figure 1. Distance Traveled to Find the Platform in MWM in Different Study Groups during Training Trials

The number of neuronal cells in the hippocampal CA1 subregion significantly decreased in CCH rats compared to the controls ($P<0.01$) (Figures 3 and 4). *D. multicaule* administration after CCH surgery significantly increased neuronal cell density. Cell density in the CCH + D. multicaule group increased significantly compared to the CCH animals ($P<0.05$) (Figures 3 and 4).

Discussion

Our study showed that *D. multicaule* extract administration improved spatial memory. The precise mechanism of this effect is not clarified but it seems that the inhibition of AChE (Acetylcholinesterase) enzyme activity by *D. multicaule* may be the main mechanism.[10,14] Cholinergic signaling in the limbic system plays an important role in cognitive processes. AChE enzyme inactivates cholinergic neurotransmission by hydrolyzing acetylcholine into choline and acetate in the synaptic cleft.[16] Substances that can inhibit AChE activity have therapeutic potential for enhancing cognitive function. Mandegary et al reported that *D. multicaule* can inhibit AChE enzyme activity.[10] Therefore, reduction of AChE enzyme activity by *D. multicaule* may be the main mechanism for cognitive improvement.

Dracocephalum multicaule can improve memory function because it can maintain long-term memory-related signaling molecules in the hippocampus of rats with vascular dementia by increasing p-CaMKII/ERK/CREB signaling transduction.[17]

Dracocephalum multicaule has been reported

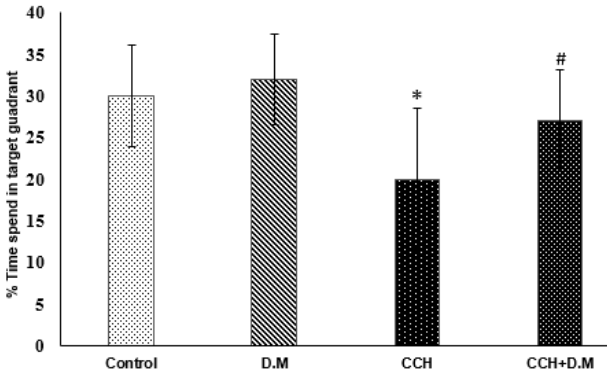

Figure 2. Percentage of the Time Spent in Target Quadrant in Different Study Groups During Probe Trials. *$P<0.01$ compared with controls. # $P<0.05$ compared with CCH.

Table 1. Latency Time and Swimming Speed in the MWM During Trail Days

	Swimming speed (CM/s)				Latency time (s)			
	D1	D2	D3	D4	D1	D2	D3	D4
Control	21.1 ± 4.1	22.7 ± 3.1	23.8 ± 1.2	24.1 ± 1.1	58 ± 6	48 ± 8	41 ± 7	28 ± 4
Dracocephalum multicaule	20.9 ± 2.2	21.1 ± 3.3	22.3 ± 1.5	23.2 ± 2.3	57 ± 5	50 ± 4	65 ± 6	27 ± 3
CCH	20.1 ± 3.1	21.8 ± 2.7	22.8 ± 2.1	22.14 ± 2.5	60 ± 8	58 ± 9	48 ± 3	34 ± 5
CCH + *D. multicaule*	20.1 ± 2.5	22.4 ± 1.7	22.8 ± 1.6	22.5 ± 2.6	60 ± 7	52 ± 4	46 ± 5	31 ± 7

MWM: Morris water maze, CCH: chronic cerebral hypoperfusion, D: day.

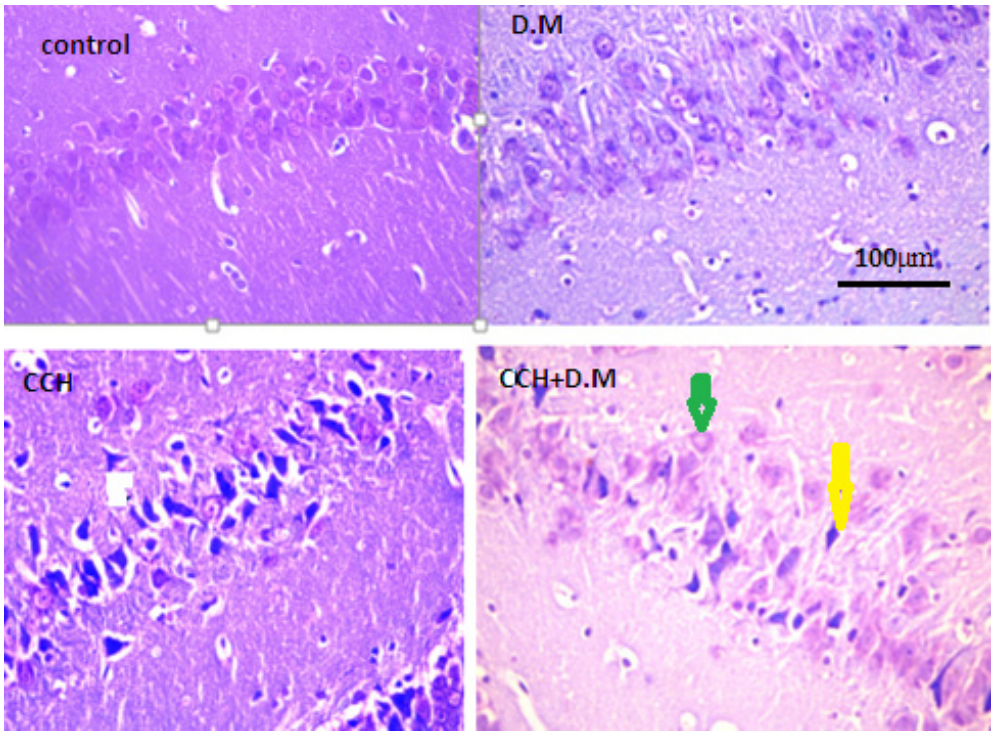

Figure 3. Staining the Hippocampal CA1 with Cresyl Violet (Magnification of 400x) in Different Study Groups. Green arrows show living neurons and yellow arrows represent dead ones. CCH: chronic cerebral hypoperfusion; D.M.: *Dracocephalum multicaule*

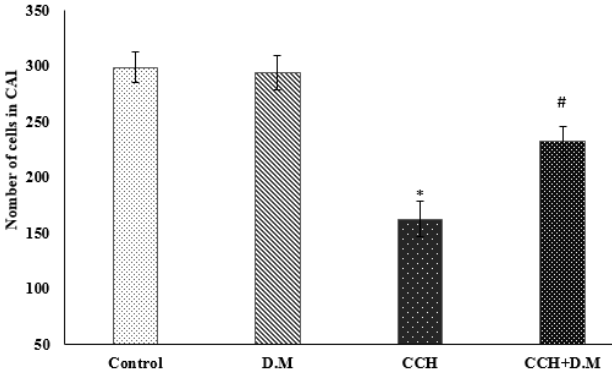

Figure 4. The Number of Neuronal Cells in Hippocampal CA1 Subregion in Different Study Groups. *$P<0.01$ compared with controls. # $P<0.05$ compared with CCH. CCH: chronic cerebral hypoperfusion; D.M.: *Dracocephalum multicaule*

to improve neurodegeneration induced by CCH. Antioxidant and anti-inflammatory effects of *D. multicaule* on the brain cortex and its ability to inhibit the apoptotic pathway in the hippocampus of 2VO rats have been reported. Pretreatment of vascular dementia with *D. multicaule* extract can reduce oxidative stress and neuroinflammation by regulating ox-CaMKII pathway in the cerebral ischemic rat.[18] In support of this finding, luteolin, a component of *D. multicaule*, has been reported to induce hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome.[19] In addition, oleanolic acid, another component of *D. multicaule*, has been reported to stimulate BDNF (brain derived neurotrophic factor) synthesis in the frontal cortex and hippocampus of the ischemic rats.[20]

Anti-oxidative, anti-inflammatory, and anti-apoptotic properties of *D. multicaule* species have been proven by several studies.[21,22] Kaempferol, apigenin, luteolin, quercetin, and oleanolic acid as major constituents of *D. multicaule* play important roles in the enhancement of cognitive function.[23-25]

Furthermore, in a previous study, it has been shown that *D. multicaule* can effectively improve memory impairment induced by scopolamine in mice. This effect is mediated by ERK-CREB signaling pathway as an important pathway in memory processing.[13]

In conclusion, this investigation showed that *D. multicaule* improved cognitive impairment caused by CCH in rats. Furthermore, *D. multicaule* extract increased neuronal cell density in CA1 reign of the hippocampus in CCH rats. These findings suggest that *D. multicaule* may be considered an effective substance for the treatment of cognitive decline related to CCH.

Limitations and Strengths

Animal death after the surgical procedure is one of the limitations of this study; however, we increased the survival rate using aseptic methods and minimized handling the vagus nerve. Visual deficit after vessel occlusion have been reported in albino rats used for all animal studies; however, no considerable effect on their behaviors has been reported. Using natural products such as *D. multicaule* for cognitive enhancement is one of the available remedies for vascular dementia.

Authors' Contribution

Conceptualization: Fatemeh Khojasteh.
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Formal analysis: Fatemeh Khojasteh.
Funding acquisition: Gharibreza Nazerirad.
Investigation: Roghayeh Roohi-Shahalibigloo.
Methodology: Fatemeh Khojasteh.
Project administration: Fatemeh Khojasteh.
Resources: Fatemeh Khojasteh.
Software: Gharibreza Nazerirad.
Supervision: Fatemeh Khojasteh.
Validation: Fatemeh Khojasteh.
Visualization: Fatemeh Khojasteh.
Writing–original draft: Fatemeh Khojasteh.
Writing–review & editing: Fatemeh Khojasteh.

Competing Interests

There is no conflict of interests related to this manuscript.

Ethical Approval

This study was approved by the Ethics Committee of Zabol University of Medical Sciences (Ethics No. 1402.077).

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