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Research Paper: The Effect of Herniarin on Spatial Working Memory, Pain Threshold, and Oxidative Stress in Ischemic Hypoperfusion Model in Rats

Zahra Nazari Barchestani[1], **Maryam Rafieirad**[1*]

1. Department of Biology, Izeh Branch, Islamic Azad University, Izeh, Iran

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ABSTRACT

Background: Ischemia causes severe neuronal damage and induces oxidative stress, memory impairment, and reduces pain threshold. Herniarin is a powerful antioxidant.

Objectives: This study aimed to evaluate the effect of herniarin on memory, pain, and oxidative stress in an ischemia model in male rats.

Materials & Methods: In this study, 50 male rats were divided into 5 groups of control, sham, ischemic, and two other ischemic groups, which received herniarin at doses of 150 and 300 mg/kg by gavage for 14 days. Behavioral tests were performed by shuttle box, and Y-maze and pain tests were performed by Tail-Flick test. Then, the rats' brains were extracted to evaluate lipid peroxidation and measure the levels of thiol and Glutathione Peroxidase (GPX) in the hippocampus and striatum tissues. The results were expressed as Mean±SEM and then analyzed using suitable statistical methods of ANOVA and least significant difference post-hoc test in SPSS V 20.

Results: Herniarin significantly increased the avoidance memory, spatial memory, and pain thresholds of ischemic rats at different concentrations ($P<0.001$). Besides, the amount of malondialdehyde (MDA) and thiol in the ischemic group increased significantly in comparison to the control group ($P<0.001$). Also, in the ischemic group, GPX ($P<0.001$) significantly decreased. Decreased MDA ($P<0.001$) and thiol ($P<0.001$) and increased GPX levels were observed with herniarin administration ($P<0.01$).

Conclusion: According to this study's results, herniarin can remove free radicals and oxidant substances from the brain. Thus, it improves memory and pain thresholds in the brain hypoperfusion ischemia model.

Keywords: Herniarin; Memory; Pain; Oxidative stress; Cerebral ischemia; Rats

*** Corresponding Author:**
Maryam Rafieirad
Address: *Department of Biology, Izeh Branch, Islamic Azad University, Izeh, Iran*
Tel:*+98 (61) 43642625;* ***Fax:****+98 (61) 43642625*
E-mail: *rafieirad.m@gmail.com*

Highlights

- Herniarin administration decreases oxidative stress in the animal model of ischemia.
- Herniarin administration increases memory and reduces pain caused by the animal model of ischemia.

Introduction

Cerebral stroke is known as the fifth leading cause of death and the leading cause of physical and mental disability in the world. Stroke is associated with a variety of complications, including cognitive impairment. During ischemia, a temporary or permanent reduction in blood flow to the brain reduces or inhibits transferring the glucose and oxygen needed to provide cellular homeostasis [1]. At this stage, processes such as cytotoxicity due to excitation, acidosis, ion imbalance, oxidative stress, lipid peroxidation, inflammation, and apoptosis cause cell death. In the next stage, reperfusion, mitochondrial dysfunction, the release of glutamate and inflammatory mediators, production of reactive oxygen species, and lipid peroxidation occur. The main event during cerebral ischemia is the production of free radicals and reactive species of oxygen and nitrogen, which due to high reactivity, damages lipids, proteins, deoxyribonucleic acid and eventually results in neuronal death. Free radicals are involved in breaking the blood-brain barrier and causing cerebral edema [2].

Over the past decade, clinical treatments have reduced mortality in the early stages of stroke. However, cognitive impairments continue to have adverse consequences for patients' quality of life. Cells in the hippocampus, which play an essential role in memory formation, are very vulnerable to cerebral ischemia. The cytokines produced in cerebral ischemia lead to the loss of pyramidal cells in the CA1 section of the hippocampus. Rebleeding also makes Reactive Oxygen Species (ROS) and damages neurons. Impaired memory and learning may be due to increased ROS and decreased levels of enzymatic and non-enzymatic antioxidants in the hippocampus [3]. Learning and memory are vital mechanisms for a living system. These brain processes allow animals to use environmental information and increase their chances of survival [4].

The hippocampus plays an essential role in the consolidation of memory and spatial learning in mammals [5]. So the destruction of this part disrupts the use of spatial memory [6]. Damage to the hippocampus causes short-term memory and spatial memory loss [7]. Also, clinical evidence implies that chronic cerebral leads to pain disorder [8], and the relationship between pain and memory impairment following stress oxidative has been established [9]. Pain is an essential part of the body's supportive system and a quick warning to the nervous system to create a motor movement to minimize physical damage [10]. Antioxidants are essential substances that can reduce the side effects of stroke and the consequences of ischemic stroke. There is a natural balance between the production of free radicals and the activity of the body's antioxidant system. Any change in this balance causes tissue oxidative stress [11].

The antigenotoxic effects of umbelliferone (UMB), herniarin (HER) and 7-isopentenyloxy coumarin (7-IP), common natural dietary coumarins, were evaluated on the human lymphocyte DNA damage using single-cell gel electrophoresis [12]. Plant-derived phenolic coumarins largely occurred in fruits and vegetables and play beneficial roles in the human body as dietary antioxidants. Simple coumarins, such as umbelliferone and herniarin, are widespread natural coumarins. They occur in many familiar plants of the family Apiaceae such as carrot, coriander and wild celery and showed promising biological properties [13, 14]. Herniarin is a methoxy analogue of umbeliferone occuring naturally in some flowering plants [15].

Herniarin was reported to have anti-dermatophytic activity [16]. As mentioned earlier, the increase in free radicals following ischemia [17] plays a major role in ischemic damage. Therefore, increasing antioxidant power by using natural antioxidants can play a useful role in reducing oxidative stress caused by the production of free radicals following ischemia [18]. This study aimed to investigate the effect of herniarin on spatial working memory, pain threshold, and oxidative stress in an animal model of hypofusion ischemia.

Materials and Methods

Animals

In this experimental study, 50 adult male Wistar rats (weight range of 250-200 g) were purchased from the

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Animal Breeding Center of Ahvaz Jundishapur University of Medical Sciences. The animals were kept in standard conditions of 20°C±2°C and 12-hour light-dark cycle (lights on 7:00 AM). They had enough access to compressed food from Pars Livestock Company in Tehran Chavdaneh Company in Shahreza, Isfahan, and to piping water from Izeh water treatment company. They were kept in standard cages in the animal Keeping Center of Islamic Azad University, Izeh Branch. The animals were trained daily for a few minutes to facilitate the work and adapt to environmental conditions and animal testing. The animals were randomly divided into 5 groups, including the healthy group without ischemic induction and without receiving medication (control), the sham group (going under surgery without cutting carotid arteries), the ischemic group with blocked common carotid arteries, and two ischemic groups which received herniarin purchased from Gol Elixir Pars Company (CAS No: 531-59-9; purity, greater than 95%; Mashhad, Iran) at doses of 150 and 300 mg/kg by gavage for 14 days [19].

Surgical method for creating ischemia

Permanent bilateral carotid artery occlusion (2-VO) is a model for permanent cerebral hypoperfusion ischemia. After one day of animal deprivation, ketamine/xylazine anesthesia (100 mg/5 mg/kg body weight) was induced. A cleft was made in the middle of the abdominal part of the neck skin, and the subcutaneous adipose tissue was removed from the thyroid. Then, the carotid artery was separated from the surrounding tissues and blocked by skin stitch tools with two tight knots around the arteries (up and down), and then the arteries were completely cut off. The animals were allowed to have water and food after regaining consciousness. After a week, a similar surgery was performed on the other side [20]. Three days after recovery, the gavage began (Figure 1).

Y-Maze test for measuring the alternation behavior

This test was used for evaluating the animal's working memory at a superficial level. Y-maze testing device was formed with three arms made of MDF. Every Y-maze arm was 46 cm long, 15 cm high, and 15 cm wide, and was placed as equilateral to each other with angles, and the arms were connected to each other through a central area (arms were marked as A, B, and C). The animal was placed in one of the three arms slowly without stress after handling, and its movements were observed for 5 minutes. After 5 minutes, the rat was gradually removed from the maze, and all of the internal surfaces of the maze were cleaned with 70% alcohol to prevent the rat's sense of smell in the arm selection. The animal with memory problems passed the arm more often. To perform this test, each rat was placed at the end of one arm, and it could reach all parts of the maze in 5 minutes. The number of times an animal entered each arm was recorded. The entry of each animal into the arm was when its back legs were completely inside the arm. The alternation behavior was considered a successful and consecutive entry (series) into all arms in three overlapping sets. Each test was repeated 3 times, and an average of 3 repetitions was recorded for each rat [21].

Passive avoidance memory

Using shuttle box device, including two chambers of dark and light with a floor which was covered with steel metal wires with 1-2 mm diameter in 1 cm distance and a device for generating electrical current. A slight shock with 75 V and an alternate current of 0.3 mA was applied to the animals' sole for 3 s only once. First, each animal was placed inside the shuttle box with an open guillotine door to get acquainted with the training device and freely move inside and outside the chamber. Then, the animal was put inside the light box, and the latency time of the animal's going to the dark box was recorded (learning). Upon entrance of the animal into the dark chamber, the guillotine door was closed, and electrical shock was applied to its sole. After 24 h (one day), the latency time of the animal's entrance into the dark chamber (which had had a shock but this time did

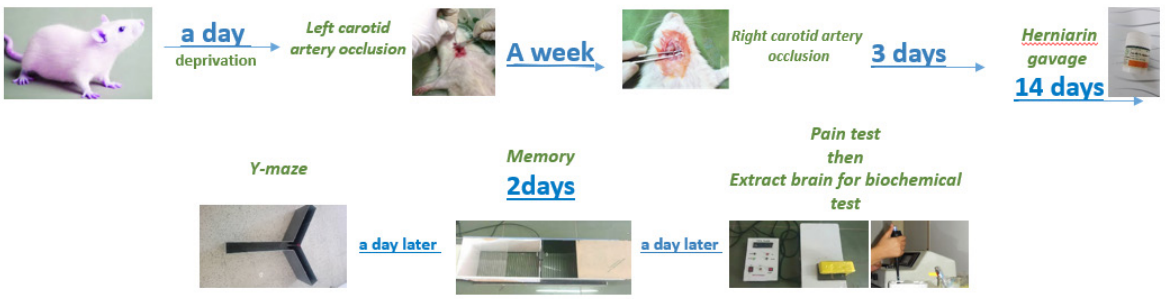

Figure 1. The experiment protocol diagram

not have any shock) was measured in terms of second as passive avoidance memory (step-through latency) [22].

Pain threshold

A Tail-Flick assay was performed using the Tail-Flick analgesia meter and radiant heat. A beam of light was focused on the dorsal surface of the tail, approximately 8 cm from the tail's tip. The intensity of the heat stimulus was adjusted so that the baseline latencies settled on 10 s. Each nociceptive test consisted of the mean value of three measurements in each rat [23].

Malondialdehyde measurement

In this test, the 7-rat groups were used, and 2 parts of the striatum and hippocampus were extracted from the head of each rat. The intended tissues were immediately weighed, and 10 g/mL of solution with a specific amount of 1.5% homogenized potassium chloride was added for every 1 g of tissue. About 0.5 mL of the homogenized solution was removed, and 2.5 mL of 3% trichloroacetic acid was added and stored for 10 min at 37°C and finally centrifuged at 3000 rpm for 10 minutes. About 0.5 mL of the upper solution was removed after centrifugation, and 0.3 mL of the phosphoric acid solution and 1 mL of 0.67% trithiobarbituric acid solution were added to each one then placed in boiling water for 45 minutes. The tubes were cooled in an ice container, and 4 mL of butanol was added to each one. After vortex, it was centrifuged for 20 minutes at 2000 rpm, and finally, the adsorption was read with a wavelength of 532 nm. After placing the spectrophotometric and adsorption numbers in the standard linear curve equation, the MDA concentration was evaluated based on (nmol/g/wet tissue) [20]. First, the standard curve had to be drawn, which required a standard MDA solution, and the absorptions were read using a spectrophotometer device, and the concentrations were measured using the linear relationship of the standard curve. About 0.5 ml of standard solution at concentrations of 8, 10, 4, 6, 2, 1, 0.5 µM was removed. Then, 3 mL of 1% phosphoric acid solution was added, and the remaining steps were performed as before. Thiol measurement is done as follows: DTNB (element reagent) was used for evaluating the thiol group. In a test tube, 1 mL of tris buffer was added (PH=8.6) to 50 µg of tissue hemogenic solution, and its optical absorption was measured at 412 nm (A1). Then, 20 mL of DTNB reagent was added to the tubes, stored at room temperature for 15 minutes, and then its absorption was measured at the same wavelength (A2). Control absorption (containing tris buffer and) was measured at 412 nm (B). The values of A1, A2, and B were placed in Equation 1, and the amount of thiol groups was calculated [20].

1. The amount of thiol groups (mM) = (A2-A1-B) × 1/07/0/05 ×13/6

Measurement of glutathione peroxidase activity

Sampling and measuring the activity level of glutathione peroxidase were carried out in accordance with the instructions provided by the BioVision Incorporated, Milpitas, CA, USA commercial kit. One unit of activity means the amount of enzyme which oxidizes 1 µmol from Nicotinamide Adenine Dinucleotide Phosphate (NADPH) to NADP+per minute under kit conditions at 25°C.

Measurement of pain threshold

Using the Tial-Flick device (made by Mr. Mohandespoor), the pain threshold was evaluated in animals in different groups. In this method, which is a standard method for measuring pain in animal models, heat at 50-55°C was flashed to a point at a distance of 8 cm from the tail of rats in and the delay time of shaking their tail from the heat was recorded. The time of cutting the heat to prevent tissue damage to the tail was adjusted and controlled for 10 s. Such an action was performed for all of the tested groups [21].

Data analysis

The results were presented as Mean±SEM and then analyzed using suitable statistical methods of 1-way analysis of variance (ANOVA) and least significant difference (LSD) Post-Hoc Test in SPSS V. 20. The difference in the results of different groups was considered significant at $P<0.05$.

Results

As indicated in Figure 2, the percentage of alternation behavior in the ischemic group decreased significantly in comparison to the control group (25±1.48) ($P<0.001$). Besides, the rate of frequency percentage in the ischemic group receiving a dose of 150 mg/kg (42.9±1.64) and a dose of 300 mg/kg (41.67±0.85) ($P<0.001$) herniarin increased significantly compared to the ischemic group.

In this study, as seen in Figure 3, evaluating initial latency time inside the shuttle box or latency of animals is going to the dark box (learning) showed a significant difference between the control and ischemic groups, which indicated decreased passive avoidance memory in the ischemic group compared to the control. Also, the treat-

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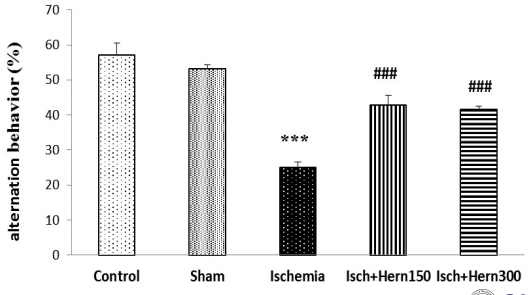

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Figure 2. Mean±SEM of the percentage of alternation (periodic) inside Y-maze between the control group, ischemia group, and the two ischemic groups orally receiving 150, 300 mg/kg herniarin for 14 days

***P<0.001 vs. control; ###P<0.001 vs. ischemic rat.

ment with a dose of 150 mg/kg herniarin (284.7±7.5) (P<0.001) and at a dose (284.4±9) (P<0.001) of 300 mg/kg in ischemic rats could significantly increase this avoidance memory.

By comparing the delay time in the Tial-Flick test in Figure 4, a significant decrease was observed in delayed response (delayed emergence of painful tail reflex) in ischemic rats compared to the control group (1.929±0.11) (P<0.001). On the other hand, this Figure shows that the delay in taking the Tial-Flick test in the ischemic groups receiving a significant dose of 300 mg/kg increased significantly compared to the ischemic group (3.414±0.12) (P<0.01). However, a dose of 150 mg/kg of herniarin failed to indicate a significant increase. MDA within the hippocampus (Figure 5A) increased significantly in the ischemic group than the control group (158±9.5) (P<0.001). Also, the level of malondialdehyde in the experimental group decreased compared to the control group, but this decrease was

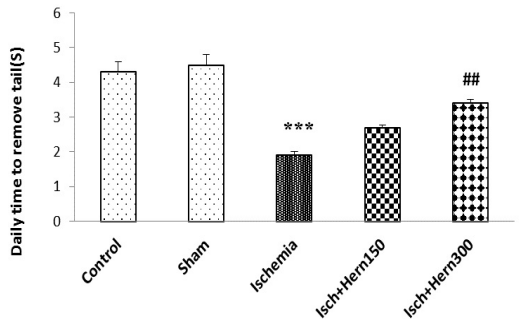

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Figure 4. Mean±SEM of painful tail reflex time inside, the Tail-Flick between the control group, ischemia group, and the two ischemic groups orally receiving 150, 300 mg/kg herniarin for 14 days

***P<0.001 vs control & ##P<0.01 vs ischemic rat.

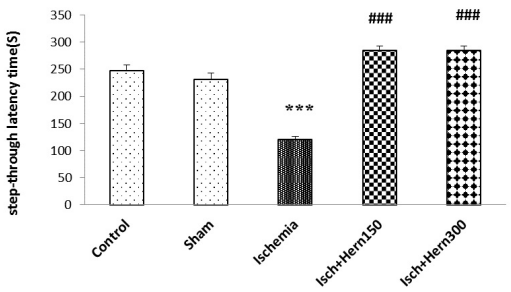

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Figure 3. Mean±SEM of step-through latency time inside the shuttle box between the control group, ischemia group, and the two ischemic groups orally receiving 150, 300 mg/kg herniarin for 14 days

***P<0.001 vs control; ###P<0.001 vs ischemic rat.

not significant. However, the ischemic group receiving a dose of 150 mg/kg (69.7±7.2) (P<0.001) and a dose of 300 mg/kg (68.1±8.7) (P<0.001) herniarin had a significant decrease compared to the ischemic group. As indicated in Figure 5B, in the striatum, malondialdehyde levels in the ischemic group receiving a dose of 150 mg/kg (114±15) (P<0.001) and at a dose of 300 mg/kg herniarin decreased significantly compared with the ischemic group (69±4) (P<0.001).

As can be observed in Figure 6A, the amount of thiol in the ischemic group receiving a dose of 300 mg/kg (0.24±0.017) (P<0.001) of herniarin decreased significantly compared to the ischemic group, but with 150 mg/kg of herniarin, the difference was not statistically significant. Figure 6B indicated that the concentration of thiol in the ischemic group increased significantly compared to the control group (0.56±0.02) (P<0.001). The ischemic group receiving both herniarin doses decreased significantly compared with the ischemic group.

The activity of glutathione peroxidase in the hippocampal tissue (Figure 7A) in the ischemic group decreased significantly compared to the control group (0.8±0.04) (P<0.001). While the activity in the ischemic group receiving a dose of 300 mg/kg (1.1±0.08) (P<0.01) of herniarin increased significantly compared to the ischemic group. The activity of the glutathione peroxidase in the striatum tissue in the ischemic group receiving a dose of 150 mg/kg (8.8±0.4) (P<0.01) herniarin and a dose of 300 mg/kg (9.1±0.8) (P<0.01) herniarin increased significantly compared to the ischemic group (Figure 7B).

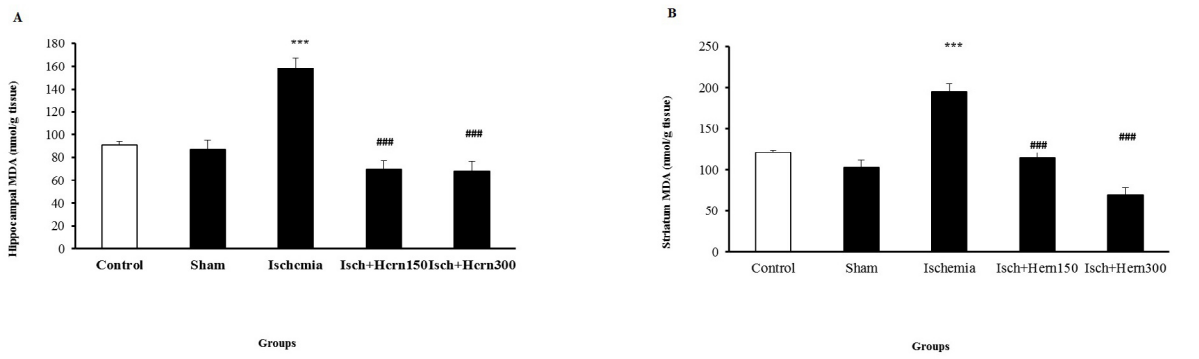

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Figure 5. Mean±SEM of concentration of malondialdehyde (MDA)

A. In the hippocampus; and B. Striatum between the control group, the ischemia group, and the two ischemic group orally receiving 150, 300 mg/kg herniarin for 14 days

***P<0.001 vs control; ###P<0.001 vs ischemic rat.

Discussion

Oxidative stress damages proteins, DNA, membrane lipids, and cellular organelles directly affects the progress of early aging, cancers, cardiovascular, neurological, and degenerative diseases [24]. The antioxidant activity of several natural compounds has been previously reported by Mosaffa et al. [25, 26]. In this study, we investigated the antioxidant activity of herniarin, which is in line with past studies [27, 28]. These compounds occur widely in daily foods, including Citrus spp, carrot, coriander, golden apple, and many other edible plants. Coumarins, a phenolic plant derivative class, are possible scavengers of reactive oxygen radicals [13]. Although there are numerous investigations regarding antitumor and other biological activities of these compounds, this is the first report on the antioxidant effects of herniarin on hypoperfusion ischemia. In our findings, the rate of Malondialdehyde (MDA) indicated a significant increase in the hippocampal and striatum tissue in the ischemic group compared to the control group.

The present study indicates a significant reduction in thiol and a significant increase in glutathione in the ischemic groups receiving different doses of herniarin compared to the just ischemic group. Additionally, the present study results indicated that herniarin concentrations of 150 and 300 mg/kg could significantly increase spatial memory and passive avoidance memory in rats under ischemia. Besides, herniarin significantly increased the delay time of the emergence of painful tail reflexes in the Tail-Flick test, while the 150 mg/kg dose of herniarin failed to indicate any significant increase in pain. Past studies have shown that vascular occlusion and its result-

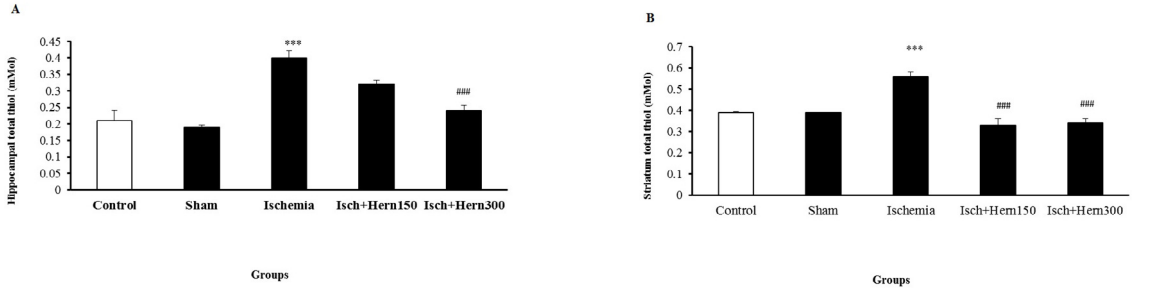

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Figure 6. Mean±SEM of concentration of thiol

A. In the hippocampus; and B. Striatum between the control group, the ischemia group, and the two ischemic groups orally receiving 150, 300 mg/kg herniarin for 14 days

***P<0.001 vs. control; ###P<0.001 vs. ischemic rat.

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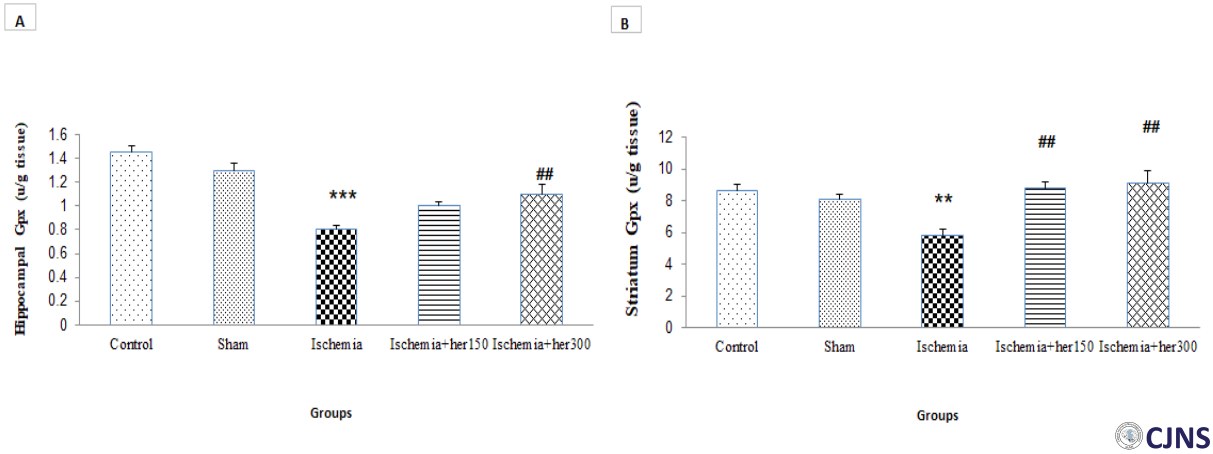

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Figure 7. Mean±SEM of activity of Glutathione Peroxidase (GPx)

A. In the hippocampus; and B. striatum between the control group, the ischemia group, and the two ischemic group orally receiving 150, 300 mg/kg herniarin for 14 days

**P<0.01 vs control; ##P<0.01 vs ischemic rat; ***P<0.001.

ing ischemia increase free radicals tenfold in the extracellular space of the hippocampus and the basal nuclei, especially in the early stages of hypoxia. Such radicals react rapidly with unsaturated fat bonds, leading to a chain reaction of peroxidase, hydro-peroxides, and aldehyde, of which MDA is one of the most important ones [29]. Oxidative stress is considered one of the diagnostic factors of reperfusion, which is effective in creating traumatic injuries [30].

The metabolism of arachidonic acid in the brain, which is in the ischemic stage, increases, which leads to an increase in free radicals [31]. Free radicals produced by the peroxidation of unsaturated fatty acids in membrane phospholipids damage neuronal membranes, cause edema, and ultimately neuronal death [32]. Thus, immediate mediation is essential to support neurons in the face of hypoxia and inflammation. Today, different drugs are used to eliminate free radicals and treat nervous disorders against ischemic injuries. Triflusal and aspirin appear to be equally neuroprotective against middle cerebral artery occlusion-induced cerebral ischemia [33]. Cells use several strategies to protect themselves from uncontrolled lipid peroxidation: inactivation of active oxygen species, trapping of eventually formed radicals, inhibition of the radical chain propagation, and repair of damaged lipids. Superoxide dismutase, catalase, and glutathione peroxidase, together with Reduced Glutathione (GSH), are the most efficient cellular agents against oxygen species and radicals. Natural antioxidants, such as vitamin E present in biological membranes, inhibit the propagation step of lipid peroxidation [34]. A study of the neuroprotective effects of the German chamomile methanolic extract (Matricaria recutita) of the plant extract significantly decreased oxidative lipid peroxidation, significantly increased antioxidant enzymes and thiol, and prevented oxidative stress and ischemia-induced neurological damage [35].

It has restorative effects on memory loss due to ischemia and may decrease pain symptoms in patients involved. The memory improvement effect of chamomile extracts maybe because of the cleansing properties of free radicals created by the active ingredients in the extract [36]. A study by Vakili et al. (2012) reported that saffron or its active ingredients could significantly decrease oxidative damage in ischemic tissues such as the brain [37]. Coumarin-dependent compounds have antimicrobial and anti-inflammatory activities [38], and such compounds have different biological properties, including antioxidant activity in laboratory conditions [16], and inhibition of cell proliferation [39].

Conclusion

The results of the present study showed that herniarin use for 14 days and once a day might improve memory impairment and reduce pain threshold by reducing oxidative stress in the animal model of ischemia. Thus, herniarin is showing its potential as a drug for the treatment of ischemia or other diseases associated with vascular disorders.

Ethical Considerations

Compliance with ethical guidelines

The study protocol was approved by the Research Committee of the Izeh branch of Islamic Azad University (No. 15330525972001) on 03.06.2018. All study procedures were done in compliance with the ethical guidelines of the 2013 version of the Declaration of Helsinki.

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Authors contributions

Conceptualization, methodology, investigation and funding acquisition: Maryam Rafieirad, Zahra Nazari Barchestani; Writing the original draft, supervision, writing, review, and editing: Maryam Rafieirad; Resources: Maryam Rafieirad and Zahra Nazari Barchestani.

Conflict of interest

The authors declared no conflict of interest.

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