

The Effects of Soy Extract on Spatial Learning and Memory Damage Induced by Global Ischemia in Ovariectomised Rats

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Submitted: 7 Sep 2013
Accepted: 5 Apr 2014

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Abstract

Background: The effects of soy extract on memory as well as the oxidative damage to brain tissue induced by ischemia was investigated in ovariectomised (OVX) rats.

Methods: The rats were divided into: 1) Sham; 2) OVX; 3) Sham-Ischemia; 4) OVX-Ischemia; 5) OVX-Ischemia-S 20; and 6) OVX-Ischemia-S 60. The common carotid artery was occluded (30 minutes), and it was then re-perfused. The OVX-Ischemia-S 20 and OVX-Ischemia-S 60 groups received 20 or 60 mg/kg of soy extract for eight weeks before the ischemia.

Results: The Sham-Ischemia and OVX-Ischemia groups took a longer time to reach the platform while, spent a shorter time in the target quadrant (Q1) than the Sham and OVX. The escape latencies in the OVX-Ischemia-S 20 and OVX-Ischemia-S 60 groups were lower while, time spent in the Q1 was higher than that of the OVX-Ischemia. In the rotarod test, there were no significant differences between the groups. The hippocampal concentrations of malondialdehyde (MDA) in the Sham-Ischemia and OVX-Ischemia groups were higher than the Sham and OVX. Pre-treatment by 20 and 60 mg/kg of the extract reduced the MDA.

Conclusion: It is suggested that soy prevents memory impairment and brain tissue oxidative damage due to ischemia in OVX rats.

Keywords: soy, ischemia, ovariectomy, rat, memory, learning, malondialdehyde

Introduction

Stroke is the third leading cause of death and one of the most important factors of disability in the world (1,2). Transient global ischemia occurs due to cardiac arrest, cardiac surgery, extreme bleeding, a near drowning, and carbon monoxide toxicity, and a large number of people experience it each year (1–3).

Global ischemia induced experimentally by the occlusion of the carotid artery leads to a reduction in the oxygen supply to the brain, brain cell death, and functional and structural damage to different brain regions, including the hippocampus (4,5). In global ischemia,

the damage is considered to be due to several mechanisms such as excitotoxicity, metabolic toxicity, oxidative stress, increased intracellular calcium, and reactive oxygen species (ROS) (6,7). The central nervous system (CNS) tissues contain a high level of membranes and fatty acids (8). It has also been shown that the susceptibility of membrane lipid constituents in the CNS to oxidative injury is very high (9,10). Moreover, it has been well documented that oxidative damage plays an important role in the pathogenesis of various CNS disorders and neurobehavioral impairments (11,12). Stress-induced lipid peroxidation affects learning and memory performance in rats (13). Conversely, antioxidants

have been shown to prevent memory impairment in various experimental models (14,15). The most vulnerable area to oxidative damage of the brain is the hippocampal CA1 region, which is largely associated with spatial memory in rats and humans (16–18).

Malondialdehyde (MDA), the final product of lipid peroxidation, is well known as a marker of oxidative stress and the antioxidant status (19). It has been well documented that lipid peroxidation occurs after brain ischemia and reperfusion (20–22). In the present study, the four-vessel occlusion model of transient ischemia which produces lipid peroxidation in brain regions including hippocampus was used (23).

Much data have shown that estrogen and progesterone are effective in neuronal survival after brain injury (24). Especially the neuroprotective effects of estrogen have been well documented (25,26). The memory protective effects of estrogen in several animal models have been reported (27). The beneficial effects of estradiol against neuronal damage in animal models of cerebral ischemia have also been reported (28). Ovarian steroid hormones have been shown to inhibit apoptotic pathways while, activating the anti-apoptotic mechanisms, and interact with growth factors to reduce excitotoxicity and increase neuronal survival after brain injury (29,30). Results of studies indicate that the administration of both estradiol and estrogen receptor agonists before a brain ischemia reduces neuronal death in the hippocampus and increases the number of intact synapses in the brain of ovariectomised rats (31–33). Interestingly, the antioxidant actions of estrogens have long been recognised in a variety of in vitro and in vivo models (34). Estrogen has been found to protect against a wide range of toxic insults, including free radical generators (34,35) and excitotoxicity (36,37). The beneficial effects of estrogen on learning and memory have also been frequently attributed to the protective properties of estrogen against oxidative damage (38).

Phytoestrogens are plant-derived molecules similar to estrogen in structure. These compounds have a two-phenolic structure that connects directly to estrogen receptors. They also seem to have antioxidant effects (39–41) and provide neuronal protection against the toxic effects of glutamate, thapsigargin, and β -Amyloid (42–46). They also protect the nerve cells against Parkinson's disease, hypoxia, and focal ischemia as well as in kainic acid-induced seizure model (47–52).

The aim of the present study was to evaluate the effects of soy extract on spatial learning and

memory and brain tissue oxidative damage induced by global ischemia in ovariectomised rats.

Materials and Methods

Plant extracts

Soy was procured from Gorgan City, Golestan Province, in the north of Iran, and was scientifically identified by the Department of Botany of Ferdowsi University of Mashhad, Iran, and the voucher specimen of the soybean was deposited. To prepare the hydroalcoholic extract, 50 g of the crumbled, dried plant was extracted with 300 mL ethanol-water (70/30, v/v), using a rotary vacuum evaporator in order to reduced to the dryness of the extracts (53,54).

Animals and experimental groups

Female Wistar rats weighing 200–250 g were kept in the animal house of Mashhad University of Medical Sciences, Mashhad, Iran, under standard conditions with a 12 h light/dark cycle and at room temperature (24 ± 1 °C). They had free access to food and water. The animal groups (n = 6–8) were: (1) Sham: The animals in this group underwent anesthesia, but the ovaries were not removed. In this group, global ischemia was not carried out. (2) Ovariectomised (OVX): The animals in this group were ovariectomised, but global ischemia was not carried out. (3) Sham-Ischemia: The animals in this group underwent anesthesia, but the ovaries were not removed. In this group, global ischemia was carried out. (4) Ovariectomised-Ischemia (OVX-Ischemia): The ovaries of the rats in this group were removed under anesthesia. The animals were then anesthetized after 8 weeks and a global ischemia was carried out. (5) Ovariectomised-Ischemia-Soy 20 (OVX-Ischemia-S 20): The ovaries of the rats in this group were removed under anesthesia. The animals received 20 mg/kg of soy extract daily for eight weeks. Then they were anesthetised and the global ischemia was carried out (5). Ovariectomised-Ischemia-Soy 60 (OVX-Ischemia-S 60): The rats in this group had their ovaries removed under anesthesia. The animals received 60 mg/kg of soy extract daily for eight weeks. Then they were anesthetised and global ischemia was carried out.

Ovariectomised surgery

For the ovariectomy, the animals were anesthetised with a solution of ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip). The

ovariectomy was preceded by a midline dorsal skin incision, and each ovary and a part of the oviduct was removed. After surgery, the muscles, and the skin were sutured and the animals were kept warm. In the Sham groups, the surgical procedures were exactly like those of the OVX groups, but the ovaries were not removed.

Induction of transient global ischemia

Transient global cerebral ischemia was induced using the two-vessel occlusion technique (55). Eight weeks after their ovariectomy, the animals were anesthetised by an injection of chloral hydrate (350 mg/kg, ip), the skin of the neck was then split and the carotid arteries were carefully exposed. Both carotid arteries were blocked with two little clamps for 30 minutes, and then reperfusion was carried out 3 days after surgery, behavioral tests were conducted.

Rotarod test

The rotarod test was used to measure motor resistance and coordination in the test animals. This is a test of motor coordination and motor learning (56–58). The latency to fall from a rotating rod is scored automatically. Motor coordination can be tested by comparing the latency to fall on the very first trial between treatment groups (56–59). Motor learning can also be assessed by comparing the first trial with subsequent trials and is evident as an increased latency to fall over time (60–64).

In this study, speed was increased from 4 to 40 rpm. The experimental procedure for learning and adaptation was begun 3 days before the two-vessel ischemia surgery. The rotarod test was also performed 3, 7, and 14 days after the global ischemia. The rats were placed on a turntable for a period of 300 seconds (maximum). The length of time they could maintain their balance on the turntable against the movement's strength was recorded as resistance time. The experiments were repeated 3 times for each rat, and the average was calculated (65).

Morris water maze

Morris water maze is an experimental method which is commonly used to evaluate spatial learning and memory in animal models. In this test, the rodents try to find the platform hidden beneath the water using the cues which is located on the around space (66–68). The water maze was a circular pool 136 cm diameter, 60 cm high, and 30 cm deep. The pool was filled with water (24–25 °C), and a circular Plexiglas platform (10 cm diameter, 28 cm high) was

placed inside it, 2 cm below the water surface in the central part of the southwest quadrant (69). Various visual signs were placed around the water maze pool, and a camera was mounted above the center of the pool with which the rats' motion was recorded and then transferred to a computer. The motions of the rats were analysed by Radyab software and the time latency to reach the platform was recorded.

The experiment was started three days after ischemia and performed four times per day (20 seconds interval), and each time the animal gave up in the water maze. Thus, the water maze was divided into four equal parts of north, south, east, and west. In each experiment the rats were released into the pool from one of these points. During the experiment the animal was allowed to swim freely, find the platform, and remain on the platform for 20 seconds. The animal was then taken out of the water. 20 seconds later, it was dropped into the water at another point. The time it took the animal to find the platform represented the amount of learning and memory and was recorded by camera. The animals were then dried and returned to the cage (70,71). One day after the last test, the platform was removed from the pool and each animal was allowed to swim in the pool for 60 seconds. The time spent in the target area was recorded by camera (72).

Biochemical assessment

At the end of the behavioral tests, the animals were put under deep anesthesia, and their brains were removed. Different parts of the brain such as the hippocampus, cortex, and cerebellum were isolated. The brain tissue with which a 10% homogeneous solution was to be created was homogenised with 1.5% Potassium chloride (KCL). For MDA levels, which represent lipid peroxidation, a 2 mL combination of thiobarbituric acid (TBA)/trichloroacetic acid (TCA)/hydrochloric acid (HCL) was added to the 1 mL homogeneous solution and boiled at 100 °C for 45 min until a pink complex was formed. After cooling it was centrifuged at 1000 g for 10 min. The absorbance was measured at 535 nm. The MDA concentration was calculated as follows (73). $C(m) = \text{Absorbance} / (1.56 \times 10^5)$.

Statistical analysis

All data were expressed as mean (SD) error. Normality test (Kolmogorov–Smirnov) was done at first. Data from the different groups collected over the five days were compared using the repeated measures ANOVA test with Tukeys' post-hoc between groups. The data obtained

from the probe trial was compared using one-way ANOVA and post-hoc test. Data for MDA concentrations were evaluated by one-way ANOVA and post-hoc test. Differences were considered statistically significant when $P < 0.05$.

Results

Escape latencies in the OVX group were significantly higher than in the Sham group ($F_{3,14} = 16.502$, $P < 0.01$; Figure 1). The animals of the Sham-Ischemia group took a significantly longer time to reach the platform than those of the Sham group ($F_{3,14} = 16.502$, $P < 0.01$; Figure 1). In the OVX-Ischemia group, the escape latencies were also significantly higher than in the OVX group ($F_{3,14} = 16.502$, $P < 0.001$; Figure 1). The escape latencies in the OVX-Ischemia group were also significantly higher than those of the Sham-Ischemia group ($F_{3,14} = 16.502$,

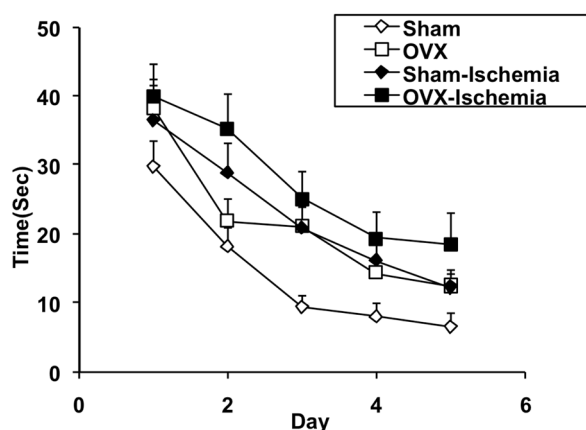


Figure 1: The effects of ischemia on time latency to reach the platform in Morris water maze (learning) of Sham and ovariectomised rats. Data are presented as mean SD ($n = 6-8$ in each group). The time latencies of the OVX group were significantly higher than those of the Sham group ($P < 0.01$). The animals of the Sham-Ischemia group took a significantly longer time to reach the platform compared to those of the Sham group ($P < 0.01$). In the OVX-Ischemia group, the escape latencies were also significantly higher than in the OVX group ($P < 0.001$). The escape latencies in the OVX-Ischemia group were also significantly higher than that of the Sham-Ischemia group ($P < 0.05$).

$P < 0.05$; Figure 1). In the probe trial, there were no significant differences in the time spent in the target quadrant (Q1) between the Sham and OVX groups ($P > 0.05$, Figure 2). However, the animals of the OVX group spent more time in the non-target quadrant (Q3) in comparison with those of the Sham group ($F_{3,14} = 3.442$, $P < 0.05$; Figure 2). The results also showed that the animals of the OVX-Ischemia group spent shorter times in the target quadrant (Q1) compared to those of the OVX group ($F_{3,14} = 2.932$, $P < 0.05$; Figure 2). In the rotarod test there were no significant differences between the Sham, OVX, Sham-Ischemia, and OVX-Ischemia groups when the animals were examined on days 3, 7, and 14 after ischemia ($P > 0.05$, Figure 3).

The results also showed that the escape latency to reach the platform in both the OVX-Ischemia-S 20 and OVX-Ischemia-S 60 groups were significantly lower than that of the OVX-Ischemia group ($F_{2,14} = 20.792$, $P < 0.001$; Figure 4). There was no significant difference between Ischemia-S 20 and OVX-Ischemia-S 60 groups ($P > 0.05$). In the probe trial, the animals of the OVX-Ischemia-S 60 group spent more time in the target quadrant (Q1) than those of the OVX-Ischemia group ($F_{2,14} = 8.354$, $P < 0.05$; Figure 5) but there was no significant difference between OVX-Ischemia-S 20 and OVX-Ischemia groups ($P > 0.05$). There was also no significant difference between Ischemia-S 20 and OVX-Ischemia-S 60 groups ($P > 0.05$). The animals of the OVX-Ischemia-S 60 group also spent shorter periods of time in the non-target quadrants (Q4) than those of the OVX-Ischemia group ($F_{2,14} = 3.562$, $P < 0.05$; Figure 5) but there was no significant difference between OVX-Ischemia-S 20 and OVX-Ischemia groups ($P > 0.05$). There was no significant difference between Ischemia-S 20 and OVX-Ischemia-S 60 groups ($P > 0.05$). In the rotarod test there were no significant differences between the OVX-Ischemia, OVX-Ischemia-S 20 and OVX-Ischemia-S 60 groups when the animals were examined on days 3, 7, and 14 after ischemia ($P > 0.05$, Figure 6).

The MDA concentrations in the hippocampal tissue of the ovariectomised animals were higher than in the sham-operated ones ($F_{5,30} = 28.24$, $P < 0.05$; Figure 7a). The hippocampal tissue concentrations of MDA in both the Sham-Ischemia and OVX-Ischemia groups were also higher than that of the Sham and OVX groups ($F_{5,30} = 28.24$, $P < 0.05$ and $P < 0.01$, respectively; Figure 7a). Pre-treatment by both 20 mg/kg and 60 mg/kg of soy extract reduced MDA concentrations in the hippocampal tissue ($F_{5,30} = 28.24$, $P < 0.05$ and $P < 0.01$, respectively; Figure 7a).

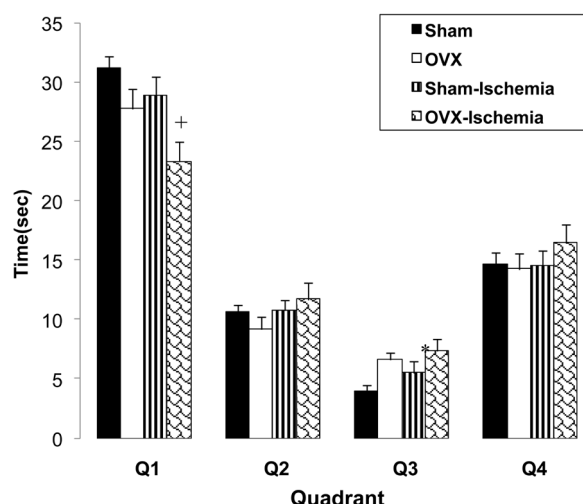


Figure 2: The effects of ischemia on the time (second) spent in each quadrant during the probe trial in Morris water maze test (memory) of Sham and ovariectomised rats. Data are shown as mean SD of 6–8 animals per group. * $P < 0.05$ compared to Sham group, ⁺ $P < 0.05$ compared to OVX group.

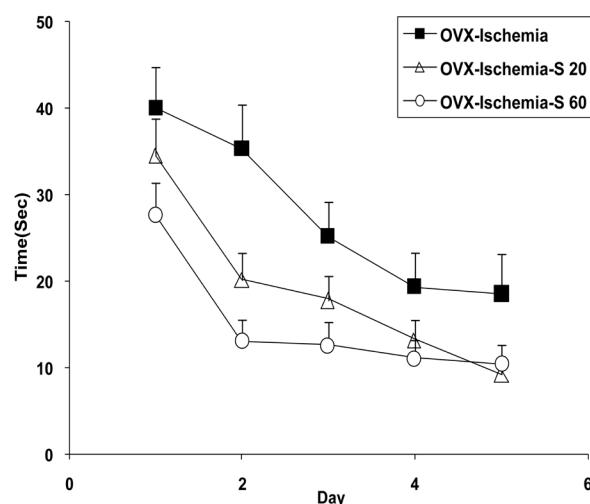


Figure 4: The effects of soy extract on impaired learning of ovariectomised rats induced by ischemia (time latency to reach the platform in Morris water maze). Data are presented as mean SD ($n = 6-7$ in each group). The results showed that the escape latency to reach the platform in both the OVX-Ischemia-S 20 and OVX-Ischemia-S 60 groups were significantly lower than that of the OVX-Ischemia group (both $P < 0.001$).

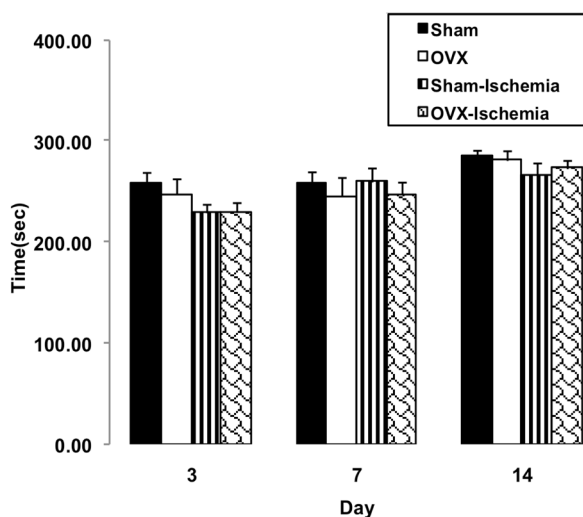


Figure 3: The effects of ischemia on rotarod test performance (motor performance) of sham and ovariectomised rats. Data are shown as mean SD of 6–8 animals per group. There was no significant difference between groups.

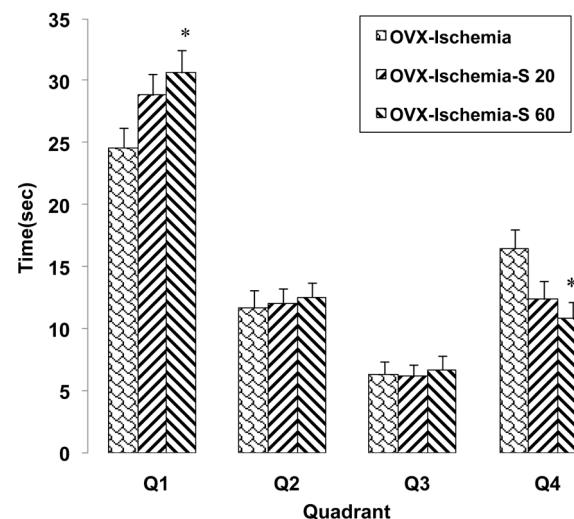


Figure 5: The effects of soy extract on impaired memory (the time spent in each quadrant during the probe trial in Morris water maze test) of ovariectomised rats induced by ischemia. Data are shown as mean SD of 6–8 animals per group. * $P < 0.05$ compared to OVX-Ischemia group.

30 = 28.24, $P < 0.01$ and $P < 0.001$, respectively; Figure 7a).

As figure 7b shows, there were no significant differences in cortical tissue MDA concentrations between the Sham-Ischemia and OVX-Ischemia groups compared to the Sham and OVX groups. There were also no significant differences between the OVX-Ischemia-S 20, OVX-Ischemia-S 60, and OVX-Ischemia groups ($P > 0.05$, Figure 7b). There were also no significant differences in MDA concentrations in cerebellar tissue between all groups ($P > 0.05$, Figure 7c).

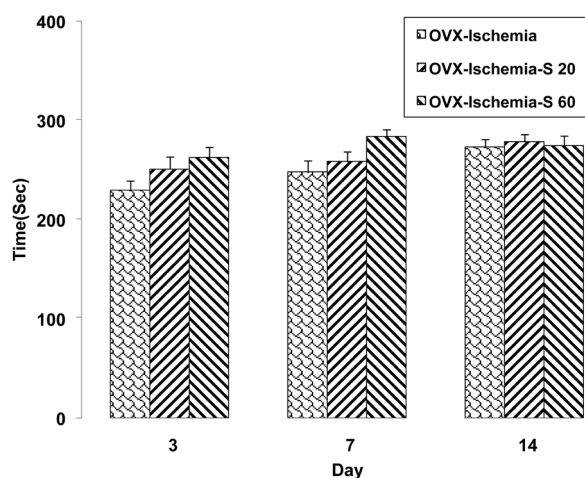


Figure 6: The effects of soy extract on impaired rotarod performance (motor performance) of ovariectomised rats induced by ischemia. Data are shown as mean SD of 6–8 animals per group. There was no significant difference between groups.

Discussion

The results of the current study showed that deprivation of endogenous ovarian hormones impaired spatial learning and memory. The escape latencies to find the platform in ovariectomised rats were higher than those in the Sham-operated ones. The ovariectomised rats also spent shorter times in the target quadrant during the probe trial, as noted by the animals' ability to recall the situation of the platform. The results were in concordance with our previous studies which experimentally showed that deletion of ovarian hormones impaired learning and memory in rats (70,74–77). However, it was found that surgical menopause has no effect on cognitive function (78). Estrogen replacement therapy has been reported to prevent or delay cognitive decline in post-menopausal women and in estrogen-depleted animals (76,78). When the estrogen levels in older female rats were low, a significant reduction in synapses was seen in the hippocampus (79).

In the present study, global ischemia impaired spatial memory in both the presence and absence of ovarian hormones. It has been suggested that during cerebral ischemia, extracellular glutamate induces a cell depolarisation, followed by an increase in intracellular calcium, which can intensify the depolarisation (80). In addition, cerebral ischemia causes abnormalities in energy metabolism, an absence of aerobic glycolysis, an accumulation of calcium and sodium ions in the cell, and the release of the excitotoxic neurotransmitters (81). It also seems that cerebral ischemia may lead to neuronal damage and cell death in the CNS, including the hippocampus which may lead to memory impairment (82).

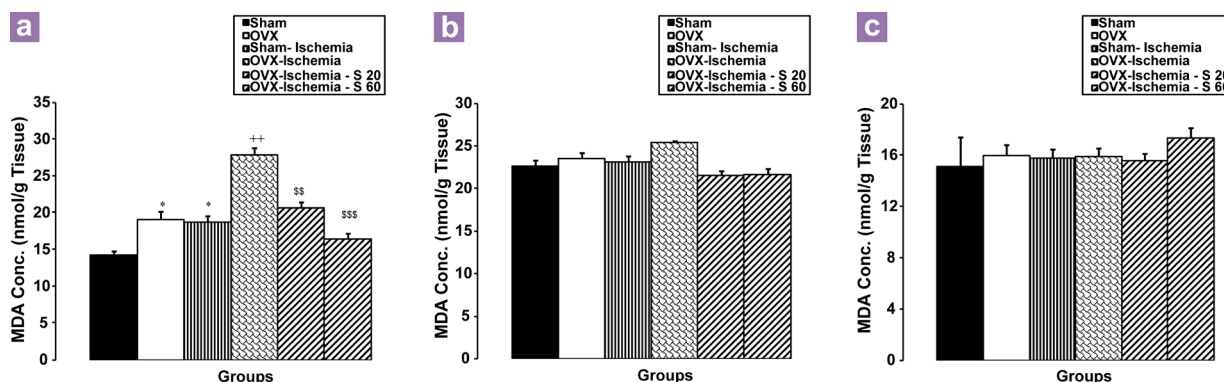


Figure 7: The malondialdehyde (MDA) concentrations in hippocampal (a), cortical (b), and cerebellar (c) tissues were compared between groups using one way ANOVA test. Data are shown as mean SD of 6 – 8 animals per group * $P < 0.05$ compared to the OVX group, ** $P < 0.01$ compared to the OVX group, *** $P < 0.01$ compared to the OVX-Ischemia group.

Regarding the neuroprotective effects of estrogens and their enhancing effects on memory which have been previously reported (29,30,83,84), it was assumed that the impairment in learning and memory due to cerebral ischemia may be different in the presence and absence of ovarian hormones. The results showed that the animals of the OVX-Ischemia group had higher latencies for finding the platform compared to other groups. They also spent shorter times in the target quadrant compared to all other groups. It does not seem that the Morris water maze impairments are due to the effects of motor movement; global ischemia did not affect the animals' performance on the rotarod test.

Jover and colleagues also showed that estradiol in young males and female Jibril had a neuroprotective effect; however, they did not find this effect in OVX female rats (29,30). Moreover, other researchers have shown that estradiol in young rodents reduced the occurrence of brain damage and death of neurons in the hippocampus caused by global ischemia (29,30,83,84).

The molecular structure of soy phytoestrogens (SPs) is similar to that of estrogen therefore, they bind to estrogen receptors and mimic their functions (85). In concurrence with this idea, it has been previously shown that soy extract mimics the proconvulsant effects of estrogen in the pentylenetetrazole (PTZ)-induced seizure model (53,54). It has also been shown that these herbal compounds have beneficial effects on memory and the cognition of both menopausal women and OVX rats (45,86). They also have neuroprotective effects *in vitro* (87,88). Furthermore, it has been shown that these herbal compounds have behavioral beneficial and neuroprotective effects against brain diseases such as Alzheimer's, Parkinson's, and cerebral stroke (42–44,89). Schreihöfer et al. also showed that dietary soy (which contains high levels of phytoestrogens) have neuroprotective effects similar to estrogen and reduce the extent of brain injury in an OVX rat model of focal cerebral ischemia (89). Thus, we hypothesized that SPs would also affect learning and memory impairment due to cerebral ischemia. Interestingly, our results demonstrated that both the low (20 mg/kg) and high (60 mg/kg) doses of soy extract improved the Morris water maze task impairments due to cerebral ischemia in ovariectomised rats; however, there were no significant differences between the groups in the rotarod test results. In fact, our results confirmed that SPs can mimic the beneficial effects of ovarian hormones on memory, especially in the

global ischemia model. Azcoitia et al. also showed that an acute administration of soy extract or genistein prevented hippocampal neuronal loss against kainic acid-induced seizures (52). Although the exact mechanism(s) are not well-known, it might be suggested that soy extract (or the phytoestrogens of soy extract) also have these beneficial effects via estrogen receptors (ERs). As mentioned above, SPs bind to ERs and mimic the actions of endogenous estrogen. Cerebral vasodilatation may be another explanation for the effects of both endogenous estrogens and phytoestrogens, which were seen in the current results (90,91). Apoptosis is also an important cause of cell death after cerebral ischemia. Ischemia causes the mitochondria to activate intracellular pathways related to apoptosis (92). Besides the prevention of excessive glutamate release, manipulation of the balance between anti-apoptotic genes and proapoptotic ones has also been suggested (29,30,93). The antiapoptotic effect of estrogen is at least in part due to the decrease in caspase-3 after global ischemia (94).

Other properties of estrogen and phytoestrogens are including the modulation of the proteins involved in synaptic plasticity and also neurotrophins (46,95). Both estrogen and phytoestrogens affect glutamate release, inhibit N-Methyl-D-aspartate (NMDA) receptors, and prevent excessive intracellular calcium which may lead to prevention of cell death due to ischemic brain injury (51,96). Many studies have pointed to the anti-inflammatory effects of estrogen and soy or its components, which play an important role in preventing cell death and brain damage in a stroke (25,97,98). Moreover, estrogen also acts as an antioxidant and helps prevent the accumulation of extracellular peroxide, the reduction of free radicals oxygen, and it limits lipid peroxidation (99). Our studies have also previously shown that an ovariectomy increases brain tissue oxidative damage criteria in rats (73). Here it was shown that global ischemia induces hippocampal tissue oxidative damage without imposing severe effects on the cerebellum or even cortical tissue. It seems that memory impairment induced by global ischemia is at least in part due to oxidative stress in the hippocampus. It was also shown that MDA concentrations in the hippocampal tissue of the soy extract-treated rats were lower than those of the non-treated ones after ischemia. The effect of soy extract on lipid peroxidation was assessed, by measuring MDA concentrations. Studies on human and on animal models using the MDA assay generally have reported an increased lipid peroxidation in ischemic brain tissues (100–102).

Therefore, the antioxidant effects of soy might be suggested as a mechanism for the beneficial effects of soy in learning and memory impairment. The results of the present study confirmed the antioxidant effects as well the beneficial effects of soy and other phytoestrogens on the nervous system functions, including learning and memory (103,104).

Conclusion

In conclusion, it was shown that global ischemia impaired the learning and memory in both the presence and absence of ovarian hormones. The results also showed that soy extract attenuated learning and memory impairments due to brain ischemia in ovariectomised rats. The antioxidant effects of soy extract might be suggested as a possible mechanism.

Acknowledgment

The authors would like to thank the Vice Chancellor of Research Affairs of Mashhad University of Medical Sciences for financial assistance.

Conflicts of Interest

None.

Funds

Vice Chancellor of Research Affairs of Mashhad University of Medical Sciences for financial.

Authors' Contributions

Conception and design and critical revision of the article for the important intellectual content: MH
Analysis and interpretation of the data: MS
Drafting of the article: FV
Statistical expertise: MAH
Administrative, technical or logistic support: HRS
Collection and assembly of data: FV, MR

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