

Neuroprotective effect of olive oil in the hippocampus CA1 neurons following ischemia: Reperfusion in mice

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ABSTRACT

Introduction: Transient global ischemia induces selective, delayed neuronal death of pyramidal neurons in the hippocampal CA1. Oxidative Stress is considered to be involved in a number of human diseases including ischemia. Preliminary studies confirmed reduction of cell death in brain following treatment with antioxidants. **Aim:** According to this finding, we study the relationship between consumption of olive oil on cell death and memory disorder in brain ischemia. We studied the protective effect of olive oil against ischemia-reperfusion. **Material and Methods:** Experimental design includes three groups: Intact ($n = 8$), ischemic control ($n = 8$) and treatment groups with olive oil ($n = 8$). The mice treated with olive oil as pre-treatment for a week. Then, ischemia induced by common carotid artery ligation and following the reduction of inflammation [a week after ischemia], the mice post-treated with olive oil. Nissl staining applied for counting necrotic cells in hippocampus CA1. Tunnel kit was used to quantify apoptotic cell death while to short term memory scale, we apply γ -maze and shuttle box tests and for detection the rate of apoptotic and treated cell, we used western blotting test for bax and bcl2 proteins. **Results:** High rate of apoptosis was seen in ischemic group that significantly associated with short-term memory loss. Cell death was significantly lower when mice treated with olive oil. The memory test results were adjusted with cell death results and bax and bcl2 expression in all groups' comparison. Ischemia for 15 min induced cell death in hippocampus with more potent effect on CA1. **Conclusion:** Olive oil intake significantly reduced cell death and decreased memory loss.

Key words: Antioxidant, hippocampus CA1, ischemia-reperfusion, neuroprotective effect, olive oil

Introduction

Hippocampus is located in inferior horn of lateral ventricles in brain's temporal lobe.^[1,2] Hippocampus plays a key role in maintaining the memory in consolidation period.^[3] Hippocampus also plays a role in organizing and storing information. It associated with feelings and memories.^[4] Damage to the hippocampus causes amnesia and loose memory later, but earlier data are maintained and hippocampus can remembers them.^[5] The remaining memory can convince us that further strengthen of the memory, make data to

transfer out of the hippocampus to other parts of the brain.^[6] Damage to the bilateral hippocampus can cause the subsequent amnesia or anterograde amnesia.^[7] Reduction the blood supply to the organ or area of the body called ischemia, which causes reduce oxygen and nutrients to tissues and organs which its result is dysfunction.^[8] Cerebral ischemia is the reduction of metabolites in brain following reduce blood flow to brain, which leading to reduced oxygen supply in it and caused cerebral hypoxia and brain tissue death or stroke.^[9,10] Hippocampus is very sensitive to ischemia and hypoxia. Hypoxia in this area causes inhibition of synaptic potentials, which is a mechanism in hypoxic cells to reduce energy consumption.^[11] Reperfusion injury refers the brain damage following return of blood flow to tissue after an ischemic period. Absence of oxygen and nutrients in blood flow creates conditions which return of blood flow to tissue cause inflammation and oxidative damage for this tissue rather than normal activities.^[12] Cerebral reperfusion causes returns oxygen

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Quick Response Code:	Website: www.ruralneuropractice.com
	DOI: 10.4103/0976-3147.112753

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to the tissue. In this situation obvious cell damage, because producing radicals and invasion supra oxide in brain tissue. This could impact on the cells and can be cause necrosis and apoptosis in neurons and other cells.^[13] Right and left common carotid artery occlusion for a few min caused ischemia in the wide area of brain and CA1 region and hillus of hippocampus are most sensitive parts prone to injury.^[14] Olive oil are made from unsaturated fatty acids.^[15] Oxidative stress is an important factor in neuronal death induced by ischemic insults. There is evidence that increased lipid peroxidation can be detected at an early stage.^[16] Antioxidants, which exist in olive oil can increases the resistance of cells against oxidation. We did the experiment on the hippocampus, due to the sensitivity of hippocampal CA1 neurons against hypoxia. In this area, neurons are extremely vulnerable against free radical accumulation. The purpose of this study was investigate of the antioxidant effects of olive oil and its therapeutic effect on reducing the mortality rate of hippocampal CA1 neurons and short term memory after induction of ischemia.

Materials and Methods

Animals

Twenty four adult male mice (Pasteur's Institute, Tehran), weighing 30-35 g at the start of the experiment, were housed three to four per cage in a temperature-controlled colony room under a light-dark cycle with free access to tap water and standard pellet food. The experimental protocol for animal care and handling was according to the guidelines of the National Institutes of Health for the use of live animals and those of the research council of Iran University of Medical Sciences (Tehran, Iran).

Experimental design

The mice were assigned as follow:

- Intact groups: ($n = 8$)
- Ischemia control groups: ($n = 8$)
- Treatment groups with olive oil: ($n = 8$)

Olive oil was given as the gavages to mice 180 micro liter/day. It was tacked one week before ischemia induction; Ischemia induced by common carotid artery occlusion (15 min) and after one week following reduction of inflammation on ischemic zone, olive oil given to treatment group for a week (180 micro liter/day). The memory test (y-maze and shuttle box) performed two weeks following ischemia induction and then brains prepared for microscopic studies that involve western blot for bax and bcl₂, Nissl staining for investigated the necrotic cell and tunnel test for investigated the apoptotic cell.

Y-maze test

This working memory test is based on spontaneous exploration and alternations between arms - neither training nor food restriction are required. Three identical arms are mounted symmetrically on an equilateral triangular center.

Mice walk between the arms and we recorded the arm name in 300 second, finally ever 3 arms name that not similar is one correct number and another are wrong number. This finding analyze with this formula: Percent

Alternation = $PA = \frac{x \times 3}{y - 2} \times 100$ (x = number of correct and y = correct + Wrong number).

Shuttle box

(Passive Avoidance - step) through this classic instrument conditioning exploits the tendency in mice and rats to escape from an illuminated area into a dark one. The instrument consists of a tilting-floor box divided into two compartments by a sliding door and a control unit incorporating a scrambler shocker.

The passive avoidance system has two compartment animal enclosures with black and white compartments, as the shuttle box system, with a sliding door partition.

In 4 days, this test in performed. In first 2 days, the mice get the habit and training with this system. Then in ternary day, they have an electrical shock (0.3 mA in 1 second) in dark room and ejected to cage. In forth day, the entrance time from laminate compartment to dark compartment was counting.

Anesthesia and monitoring

Animals will be fasted overnight and anesthetized with ketamin (Sigma Chemical Co., Saint Louis, USA) (100 mg/kg, I.P injection) and xylazine (Sigma Chemical Co., Saint Louis, USA) (10 mg/kg, I.P injection) with additional ketamin as needed (30 mg/kg,). A rectal temperature probe is placed for continuous monitoring of core body temperature. Core body temperature is maintained at $37 \pm 0.5^\circ\text{C}$ throughout the procedure by the intermittent use of a heating lamp. After anesthesia, the mice brains prepared for microscopic studies that involve tunnel test and Nissl staining and western blot for bax and bcl2.

Nissl staining

This method is used for the detection of Nissl body in the cytoplasm of neurons. This stain is commonly used for identifying the basic neuronal structure from necrotic neurons in brain and spinal cord.

Deparaffinize sections in xylene and then hydrate. Rinse in tap water and then in distilled water. Stain in 0.1% cresyl

violet solution for 3-10 min. Rinse quickly in distilled water. Then differentiate and dehydrate in alcohol then clearing and finally mount with permanent mounting medium. The Nissl body will be stained purple-blue.

Tunnel test

The TUNEL Apoptosis Detection Kit is one of GenScript's newly introduced products. The kit can detect fragmented DNA in the nucleus during apoptosis.

Deparaffinize sections with heater 60°C and xylene and then hydration. Incubate in proteinase k (30 min) then blocking the endogenous peroxidase with use of H₂O₂ in methanol in dark room then wash in tris buffer and then incubate in TUNNEL reaction mixture for 60 min in moisture condition then wash in tris buffer and then detection with incubate in POD. Then washing and use of chromogen DAB (You + 1'd this publicly. Undo3, 3'-Diaminobenzidine) in dark room then washing and use of hematoxylin for counter stain and dehydration and clearing and mount with permanent mounting medium.

Detection of bax and Bcl-2 by western blot technique

All steps were carried at 4°C. Following the ischemia reperfusion, animal were sacrificed and hippocampus tissues were removed and washed with ice-cold saline and homogenized on ice in lysis buffer containing 50 mmol/L Tris (pH 9), deoxycolate 1% for 60 seconds each, separated by a pause for 1 min at 4°C. The homogenate was incubated in lysis buffer for 30 min, with vortex-mixing every 10 min at 4°C. The homogenate was filtered and then centrifuged at 20 000 g for 20 min with a Hettich (universal 16L-, Germany) and (Universal 16L, Hettich, Germany) the resulting supernatants (lysate) were frozen at -80°C for further investigation. The protein concentration was determined by Bradford's method, using bovine serum albumin as a calibrator.

Twenty micrograms of cell lysate proteins was mixed with 5 µL of loading buffer (50 mmol/L tris, 20 g/L sodium dodecyl sulphate, 100 mL/L glycerol, 100 mmol/L β-mercaptoethanol and 0.05% bromophenol blue solution, (pH 6.8) were boiled for 5 min and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The gels were transferred to nitrocellulose membranes in Tris-glycine buffer (25 mmol/L Tris, 192 mmol/L glycine, 200 mL/L methanol, pH 7.4) for 5 h at 60 V. The nitrocellulose sheets were washed and free binding sites were saturated with 50 g/L bovine serum albumin in Tris-buffered saline buffer (50 mmol/L tris, pH 7.5, 150 mmol/L NaCl, 2 mmol/lit EDTA) for 1 h at room temperature. Then the membranes were incubated with phosphate buffer saline and mouse monoclonal anti-rat Bcl-2 antibody diluted 1:300 (by

volume) overnight at 4°C, then with rabbit anti-mouse IgG alkaline phosphatase conjugate diluted 1:500 for 90 min at room temperature. Finally, the membranes were incubated with BCIP/NBT (nitro-blue tetrazolium chloride)/ (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) alkaline phosphatase substrate solution (at room temperature) until the developed bands were of desired intensity. Then the reaction was stopped by 200 mL of 0.5 mol/L EDTA (pH 8) and 50 mL of phosphate buffered saline. Bax and Bcl-2 protein band was identified by comparing with the molecular weight marker.

Statistical analysis

All data were expressed as mean FS.E.M. For within group and intergroup comparisons, two-tailed paired and unpaired Student's T-tests were used, respectively. One way ANOVA, followed by Tukey's *post hoc* test, was used for each group at different time points. In all analyses, the null hypothesis was rejected at the level of 0.05.

Results

In this study, we used the Nissl staining to count the necrotic cell and used the tunnel kit to detect the apoptotic cell in the CA1 region. We used western blotting test for bax and bcl2 proteins.

Figures 1-3: The Nissl staining result in 3 groups. In this staining method, the necrotic cells indicated with dark and compact nucleus.

- Figure 1: The intact group without necrotic cell.
- Figure 2: The ischemic group with a lot of necrotic cell.
- Figure 3: The treatment group with infrequent necrotic cell rather than ischemic group.

Figures 4-6: Tunnel test result in 3 groups.

- Figure 4: The intact group without apoptotic cell.
- Figure 5: The ischemic group with a lot of apoptotic cell [gray cells]
- Figure 6: The treatment group with the less than other group is indicated.

Figure 7: Comparison of the density of healthy cells in the CA1 region of hippocampus. Cell density of the treatment groups is significantly different than ischemic group. [$P < 0.05$] *

Figure 8: Comparison of the short term memory (shuttle box). Ischemic group compared with the intact group represents significant difference. [$P < 0.05$] * Ischemic group compared with treatment groups represents significant difference. [$P < 0.001$] **

Figure 9: Comparison of the short term memory (y-maze). Ischemic group compared with the intact group represents significant difference. ($P < 0.05$) * Ischemic and vehicle group compared with treatment groups represents significant difference. ($P < 0.001$) **

Figures 10 and 11: Western blotting method for bax and bcl2 protein. Bax in the ischemic groups significantly

expressed but in the other groups shows less expression. Expression of Bcl2 in the treatment groups showed the success in the treatment.

Figures 12 and 13: Pixel counting result of western blot. Bax in the ischemic group was expressed significantly than other groups and expression of Bcl2 in treatment group was significantly rather than other group. ($P < 0.05$) *

Cresyl violet staining showed the situation of healthy and necrotic cells in tissue sections. The animals treated with

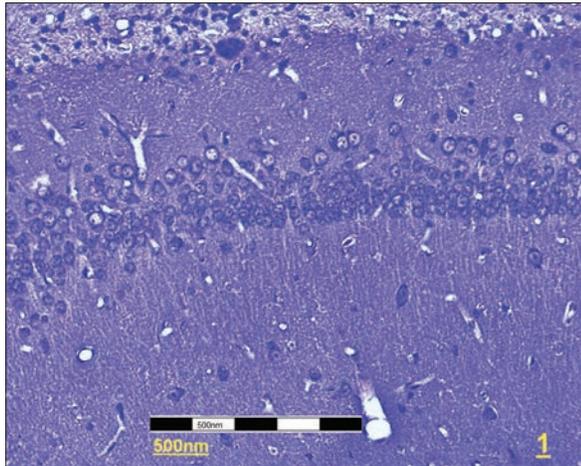


Figure 1: Nissl-intact

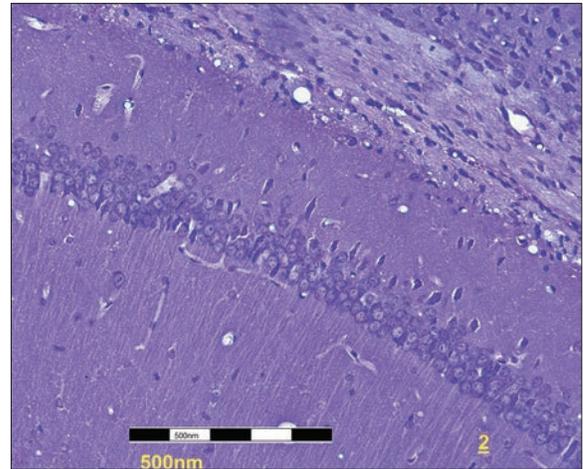


Figure 2: Nissl-ischemia

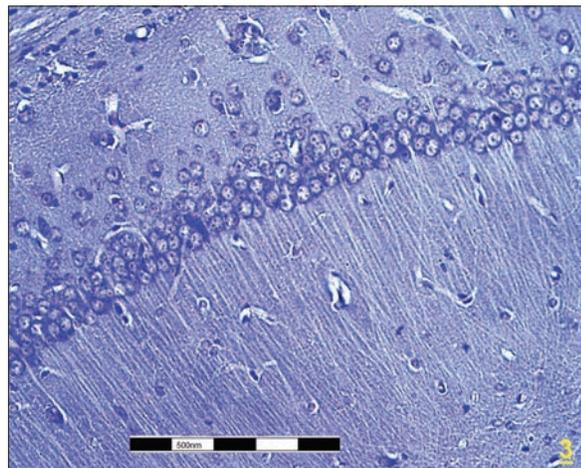


Figure 3: Nissl-treatment

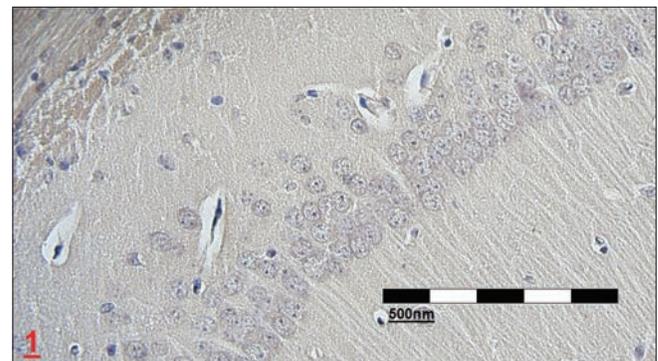


Figure 4: Tunnel-intact

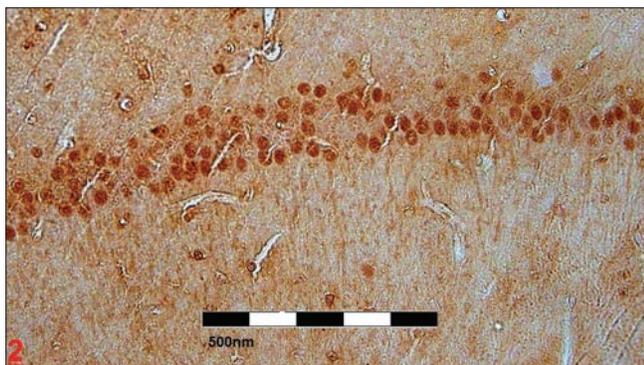


Figure 5: Tunnel-ischemia

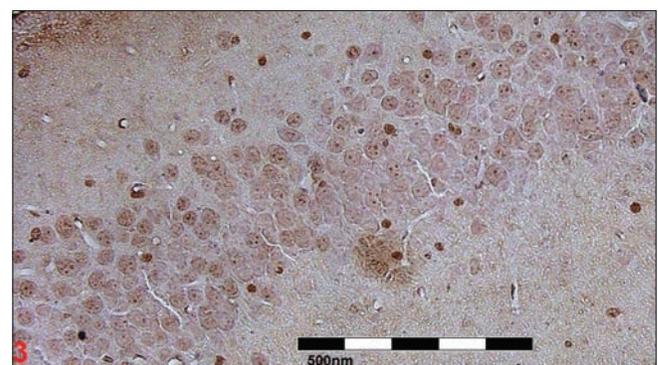


Figure 6: Tunnel-treatment

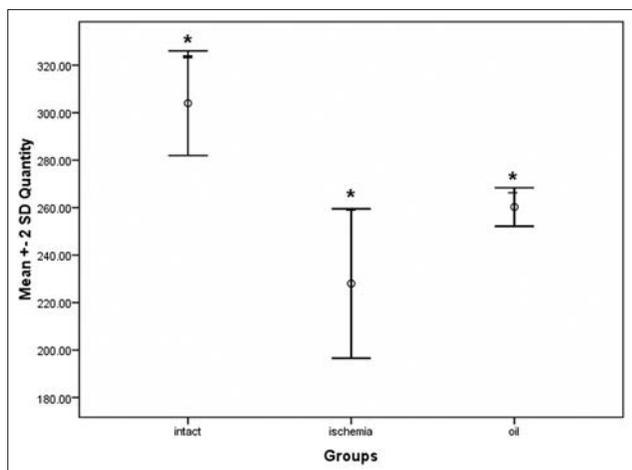


Figure 7: Cell counting diagram

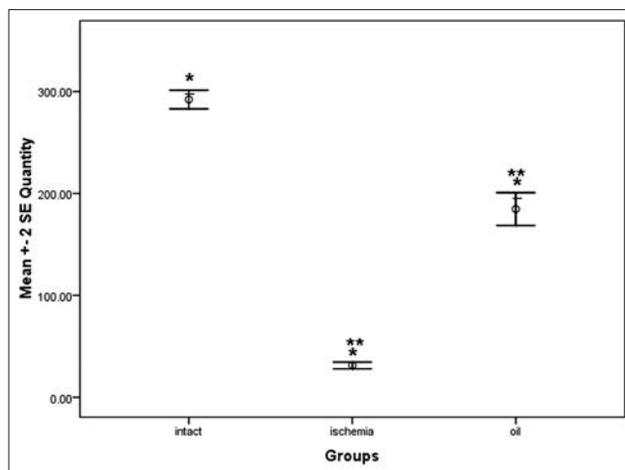


Figure 8: Shuttle box diagram

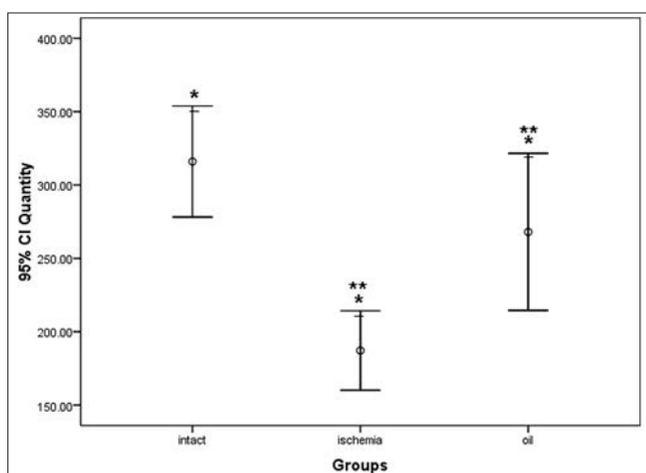


Figure 9: Y-maze diagram

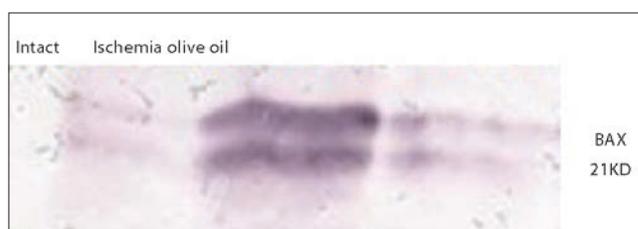


Figure 10: Bax band



Figure 11: Bcl2 band

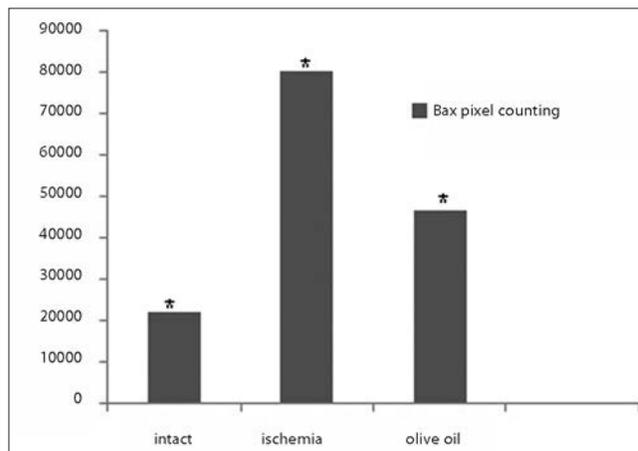


Figure 12: Bax pixel counting

olive oil had less death cells and had more cell density compared to the vehicle and ischemia groups.

Y-maze and shuttle box behavioral tests showed that ischemia leads to severe damage to short-term memory. Best results achieved by treatment with olive oil. In this group because of antioxidants in the olive oil, we can see some improvement sign. In intact group this disorder failed to record.

Tunnel test that use for detect the apoptotic cell, showed increase of this cells in ischemic group and decrease

of this cells in the treatment groups. Although, in the Western blotting result for bax and bcl2 listed below. High bax expression in the ischemic group and reducing its expression in the treated group was clearly demonstrated. Anti apoptotic protein Bcl2 in the treatment groups showed much greater expression in ischemic groups, but its expression was very low.

Discussion

Structural change in the artery wall, especially in the vital organs like the brain as result formation of atheroma

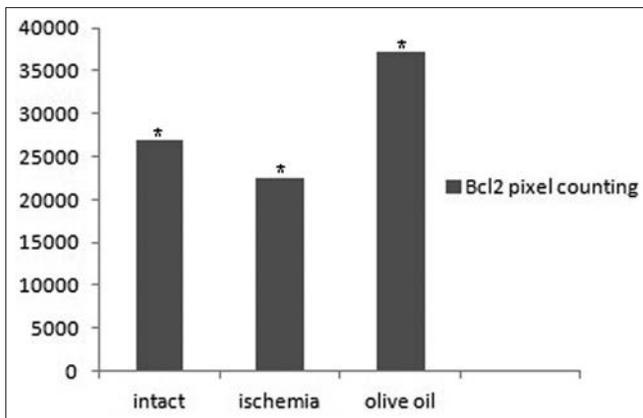


Figure 13: Bcl2 pixel counting

plaque, which reduced blood flow to the brain and causes permanent damage and increases stroke risk.^[17] When cerebral ischemia is induced experimentally in mice brain, occur changes in behavior and movements of the mice rather than before ischemia, this caused by numbers of cell death in central nervous system.^[18] We did the experiment on the hippocampus, due to the sensitivity of hippocampal CA1 neurons against hypoxia. These neurons are extremely vulnerable against free radical accumulation, following ischemia - reperfusion in this area. The antioxidant effect of virgin olive oil in the brain of healthy rabbits has been reported previously. Some components of virgin olive oil, specifically polyphenols and vitamin E, have clear antioxidant effects in some cells and tissues.^[19] Consumption of olive oil and hydrogenated oil in rabbits was associated with significant reduction in blood triglyceride levels.^[20,21] This is probably due to the presence of antioxidants in it and due to its other beneficial effects that are in the olive oil too; uses olive oil in diet could be involved in community health.^[22] The purpose of this study was investigate of the antioxidant effects of olive oil and its health impact on reducing the mortality rate of hippocampal CA1 neurons after induction of ischemia. We observed decrease cell death in the hippocampus CA1 of the treatment group with olive oil. It is due to the effects of antioxidants and unsaturated fats found in oils such as polyphenols, oleic acid, linoleic acid, linoleic acid, and vitamin E. These compounds are absorbed from the gastrointestinal tract into the blood vessel, and then some of it reaches in to the brain and past from blood-brain barrier due to being soluble in fat and dash into different parts of the brain including the hippocampus. Scientists propose frequently use of olive oil to prevent memory impairments following ischemia probably due to its antioxidant effect.^[23] Since the cholinergic, dopaminergic, serotonergic system have a important role in learning and memory^[24] however, from the compounds identified

in olive oil, which modulates of brain cholinergic system activity, this oil can help to the memory and learning.^[25] In hippocampus area the polyphenols and synergistic effects of these compounds with vitamin E causes the potent antioxidant effects on free radicals and destructive acids that are gathered due to reduction in blood supply. They can prevent from reaction between membranes; lipids with free radicals via react with this reactive radical and neutralize them. Subsequent reperfusion worsens this oxidative stress, potentiating ischemic injury. Diets rich in antioxidants might thus offer neuroprotection in cases of ischemic stroke. Antioxidant to prevent conditions in which oxidative stress may play an etiological role.^[26] A key component of this diet is olive oil, which contains monounsaturated fatty acids (MUFA) and polyphenols, compounds with a clear antioxidant effect.^[27] Ischemia - reperfusion reduces the oxygen level inside the cell that disrupts the normal cell metabolism and without the rapid supply of oxygen, the cells dies. Large amounts of free radicals in the reperfusion phase can invade and take it to death. The total consumption of olive oil with scavenge the free radicals, reduces the number of damaged cells. This will prevent the expression of pre-apoptotic bax protein and else anti apoptotic bcl2 protein expression is increased [Figure 7]. The result of this intracellular activity is inhibition process of apoptosis and cell death that subsequent reduction of ischemic complications.

Conclusion

Treatment with olive oil can be a pharmaceutical approach to lessen effects of ischemia reperfusion on hippocampus.

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How to cite this article: Zamani M, Hassanshahi J, Soleimani M, Zamani F. Neuroprotective effect of olive oil in the hippocampus CA1 neurons following ischemia: Reperfusion in mice. *J Neurosci Rural Pract* 2013;4:164-70.

Source of Support: Nil. **Conflict of Interest:** None declared.