The effect of pre-treatment with olive oil on TNFR1/NF-κB inflammatory pathway in rat ischemic stroke model

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Abstract

Introduction: Ischemic stroke is a serious neurological disease and a leading cause of death and severe disability in the world. A key component of the Mediterranean diet is olive oil, which contains compounds with antioxidant and anti-inflammatory effects. In this regard, the aim of the present study was to investigate the effect of olive oil on the ischemic damages and the inflammatory pathway of TNFR1/NF-κB in various regions of the rat brain.

Methods: In this experimental research 58 male Wistar rats were totally divided into six groups including sham, control (intact), control (middle cerebral artery occlusion, MCAO), and treatments. The intact group received distilled water, while the treatment groups received different doses (0.25, 0.50, and 0.75 ml/kg) of olive oil by gastric gavage for 30 days. Two hours after the last gavage, the rats were subjected to 60 min MCAO surgery. Twenty four hours later, the neurologic defects scores, infarct volume (in total, cortex and striatum of hemisphere) and the inflammatory factors protein expression were evaluated separately. Data were analyzed by kruskal-wallis and two-way ANOVA tests.

Results: The olive oil 0.75 ml/kg-received group displayed a significant reduction in the infarct volume, the neurological scores and the inflammatory factors protein level in comparison to the control group. Moreover, this significant difference was observed in the cortex and striatum.

Conclusion: The present results demonstrated that the neuroprotective effects of the olive oil could improve ischemic injuries. It seems that its positive impacts are partly attributed to anti-inflammatory effects of the olive oil.

Keywords: Olive oil; Infarct volume; Neurologic defects; MCAO; Stroke

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Introduction

Stroke or cerebrovascular attack is characterized by sudden disruption of blood supply to the brain which incurs serious consequences such as cerebral infarction and neurological deficits. Stroke is the second leading cause of mortality and long-term disability in developing countries including Iran (Bacigaluppi et al., 2008). Eighty five percent of strokes are caused by the cerebral ischemia (Mergenthaler et al., 2004). The useful information about the pathophysiology of cerebral ischemia has been achieved through the study of animal models. These pathophysiologic processes consist of excitotoxicity-mediated calcium overload, mitochondrial dysfunction, oxidative stress, inflammation and apoptosis (Doyle et al., 2008).
Inflammation is determined as the most overcoming process in the development of ischemic damages which its cascade is initiated approximately some minutes after the beginning of ischemia. The destructive inflammatory process is defined with up-regulation of cytokines, particularly tumor necrosis factor receptor1 (TNFR1) and nuclear factor kappa B (NF-κB), chemokines, free radicals, and microglial activation in the early phase and leukocytes rolling, the adhesion of leukocytes to endothelia and migration into the brain parenchyma in the delayed phase (Jin et al., 2010). Among cerebral ischemia-related cytokines, TNF-α and NF-κB pathway is known as the trigger of the inflammation and the cell death (Chen and Goeddel., 2002).

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that is up-regulated after a brain injury and takes part in the cell death process (Bigdeli et al., 2008). TNFR1, the most important TNF-α receptor, is expressed in most cells and the biological activity of the TNF-α is associated with TNFR1. Binding of TNF-α to TNFR1 activates NF-κB that plays a role in the regulation of inflammation and triggering apoptosis. NF-κB is a protein complex that controls the transcription of DNA, cytokine production and cell survival. Inhibition of NF-κB activity can be a promising molecular objective for improving the brain damages and reflowing the blood in the brain during cerebral ischemia (Castillo et al., 2010).

The lowest incidence of cardiovascular disease, cancer, obesity, diabetes and oxidative stress-related diseases are observed in the areas with Mediterranean diet (Khanjani Jelodar and Bigdeli, 2013). Olive oil is the main source of fat in this kind of diet. Olive oil is rich in glycero1, pigments, aromatic compounds, sterols, tocopherols, phenols, small amount of free fatty acids and resin compositions (Boskou, 2015). Increased antioxidant capacity and reduction of pro-inflammatory and pro-thrombotic mediators has been reported by two key phenolic component of olive oil (tyrosol and hydroxyl tyrosol) in the experimental animals (Fki et al., 2007). Moreover, pre-treatment of oleuropein (the important phenolic component of the olive oil) promoted neuronal survival of CA1 hippocampus in the cerebral ischemia (Zamani et al., 2013). Scavenging ability of superoxide anions and hydroxyl radicals has also been demonstrated by oleuropein (Pérez-Jiménez et al., 2006). Consequently, the previous findings indicated that the administration of olive oil induces ischemic tolerance (Pérez-Jiménez et al., 2006). Therefore, the neuroprotective properties of olive oil makes it a reliable candidate for the present research. Accordingly, it has been attempted to investigate the effect of olive oil on ischemic injuries and the involvement of TNFR1 and NF-κB pathway in these effects.

Materials and methods

Experimental protocol

Fifty eight male Wistar rats (body weight 200-300 g) were housed under conditions of controlled temperature (22±2 °C) and constant humidity with 12 hour light/dark cycle. Food and water were available ad libitum. All experimental procedures were approved by the institutional animal care and committee at Shahid Beheshti University, Tehran, Iran.

The animals were divided into four groups of seven rats including control, middle cerebral artery occlusion (MCAO) and treatment groups for the assessment of infarction volume and neurological deficits. Six groups of five rats including control, MCAO, sham and treatment groups were assigned for the evaluation of inflammatory factor’s protein levels. To study the stress of surgery, the sham group was categorized in the animal groups. Due to the fact that following the surgical stress, the related molecular pathways are activated, we had in mind to measure the degree of intervention of these pathways in our molecular studies. The control (MCAO) and control (intact) groups were considered in order to study the pathologic conditions and the physiologic baseline. Ischemic surgery was carried out on the control group (MCAO). This group was treated with distilled water orally, while the treatment groups received 0.25, 0.5 and 0.75 ml/kg/day gastric gavage of the virgin olive oil for 30 days (Mohagheghi et al., 2010). The doses were chosen according to the previous studies (in a research in which it was shown that the mean amount of olive oil consumption in the typical Mediterranean diet is 46 g/day (range: 12.6-113.1 g/day) (Rabiei et al., 2013). Two hours after the thirtieth day of gavage, the animals of the main groups were divided into two subgroups (the ischemic and the intact subgroups). The ischemic subgroups were subjected to 60 min of middle cerebral artery occlusion. Twenty-four hours
later, evaluation of neurobehavioral deficits score and then infarct volume were performed (n=7). The intact subgroups and five rats of control group (MCAO) were assigned for the evaluation of TNFR1 and NF-κB expression by the western blotting technique (n=5). The sham group underwent ischemic surgery procedure, without introducing the filament.

**Focal cerebral ischemia**
The rats were anaesthetized with chloral hydrate (Merck, Germany) at the dose of 400 mg/kg (Rabiei et al., 2013). MCAO was performed as described by Longa et al. Briefly, under a microscopic surgery, a 3-0 silicone-coated nylon suture was introduced through the common carotid artery. The filament was advanced into the internal carotid artery beyond the carotid bifurcation until the mild resistance indicated that the tip was lodged in the anterior cerebral artery and blocked the blood flow to the middle cerebral artery. Reperfusion was started by withdrawing the suture after 60 minutes of ischemia. Rectal temperature was monitored (Citizen-513w, CITIZEN) and maintained at 37 °C by surface heating and cooling during surgery.

**Neurobehavioral evaluation**
Neurological deficits scoring were performed by observers who were blind to the details of the animal grouping 24 hours after the reperfusion. Standards of neurological deficits severity scores consist of five items with 0 as minimal and 18 as maximum mark (Long et al., 2013). Score explanation was provided

Table 1: Total Neurological score

<table>
<thead>
<tr>
<th>Item</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>Raise the tail (normal=0-maximum=3)</td>
<td>Motor function (normal=0 - maximum=3)</td>
<td>Sensory function (normal=0 - maximum=2)</td>
<td>Beam test (normal= 0 maximum=6)</td>
<td>Reflex activity (normal=0 maximum=4)</td>
</tr>
<tr>
<td>middle line less than 10 (0 score)</td>
<td>Normal working (0 score)</td>
<td>Algesia and heat sensory (1 score)</td>
<td>Stand stably on the beam (0score)</td>
<td>Corneal reflex (1score)</td>
<td></td>
</tr>
<tr>
<td>Forelimb crooked (1 score)</td>
<td>Did not walk along a straight line (1 score)</td>
<td>Proprioception (2 scores)</td>
<td>Hold on to one side of the beam (1 score)</td>
<td>Auricle reflex (1score)</td>
<td></td>
</tr>
<tr>
<td>Hind limb crooked (1 score)</td>
<td>Rotatedto the hemiplegicside (2 scores)</td>
<td>-</td>
<td>One limb falling down from the beam (2scores)</td>
<td>Startle reflex (1score)</td>
<td></td>
</tr>
<tr>
<td>- Head deviated from the middle line less than 10° within 30 seconds (1 score)</td>
<td>Fall down to the hemiplegic side and could not walk (3 scores)</td>
<td>-</td>
<td>Two limbs falling down from the beam, or rotated on the beam, lasting more than 60 seconds (3scores)</td>
<td>Dysmyotonia (1score)</td>
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<tr>
<td>- - - -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sustain on the beam for 40 seconds and fall down (4 scores)</td>
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<tr>
<td>- - - -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sustain on the beam for 20 seconds and fall down (5 scores)</td>
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<tr>
<td>- - - -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sustain on the beam for 10 seconds and fall down (6 scores)</td>
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Assessment of the infarct volume

Rats were sacrificed and the brains rapidly removed and cooled in saline at 4 °C for 15 min. 2-mm thick coronal sections were then cut (Brain Matrix, Iran). The slices were immersed in 2% 2, 3, 5-triphenyltetrazolium-chloride solution (TTC) (Merck, Germany) and kept at 37 °C in a water bath for 15 min. The slices were then digitally photographed (Nikon, D40x digital) connected to a computer. Unstained areas were defined as infarct and were measured using image J software (Image J, version 1.46r). Calculation of the infarct volume was done by measuring the unstained and stained areas in each hemisphere slice, multiplying by slice thickness (2 mm) and then summatming all of the eight slices according to the method of Swanson et al. Evaluation of the infarct volume in the cortex and striatum regions was also carried out separately (Swanson et al., 1990).

Corrected infarct volume= left hemisphere volume – (right hemisphere volume – infarct volume)

Western blot analysis

Anesthetized rats were perfused transcardially and then decapitated for measurement of TNFR1 and NF-kB protein expression in the cortex and striatum regions. The tissues were homogenized by ultrasound homogenizer (4000 rpm) in 4 volumes of homogenization buffer containing 0.5% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 0.03% EDTA, NP40 (0.1%), 1 tablet protease inhibitor cocktail (Roche) and 50 mM Tris-HCl with pH 8.0. The homogenate was centrifuged at 12,000 g for 20 min at 4°C and the supernatant was collected. Homogenates were loaded together with a protein ladder (Thermo Scientific) for separating mixtures of proteins based on their size in 10% SDS-PAGE (polyacrylamide gel electrophoresis). Proteins were

![Fig.1](image1.png)

**Fig.1.** The graph shows that the effect of different doses of olive oil (0.25, 0.5 and 0.75 ml/kg/day) on total neurological deficits scores. Each column represents the mean±SEM (n=7) (kruskal-wallis test).

![Fig.2](image2.png)

**Fig.2.** The effect of various doses (0.25, 0.5 and 0.75 ml/kg/day) of olive oil on infarct volume in different experimental groups in total, cortex and striatum hemisphere of rat brain. The decreasing effect of olive oil at dose of 0.75 ml/kg/day was observed in total, cortex and striatum areas. Values are expressed as the mean±SEM (n=7). P<0.05 compared with control group (Two-way ANOVA test).
transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore). Blots were blocked at a room temperature for 75 minutes in the blocking buffer and subsequently incubated with specific primary polyclonal rabbit antibody to NF-κB (1:500 dilution; Santa Cruz), goat anti-TNFR1 polyclonal antibodies (1:500 dilution; Santa Cruz) and rabbit anti-β-actin (1:1000 dilution; Santa Cruz). They were then incubated with secondary antibodies including goat anti-rabbit and rabbit anti-goat (1:500 dilution; Santa Cruz) separately. NF-κB and TNFR1 immuno-reactive proteins were detected with advanced chemiluminescence (Enhanced Chemiluminescence, Amersham Biosciences) and film exposure. After scanning and transferring the images to the computer, signal bands were quantified by image J software. The densitometric analysis of the bands after the normalization with β-actin as a loading control was performed (Nourshahi et al., In press).

Statistical analysis
The neurological deficits score were compared using non parametric Kruskal-Wallis analysis of variance on ranks followed by the Dunn test (spss 22.0). Infarct volume, data from TNFR1 and NF-κB were analyzed by using two-way analysis of variance (SPSS 22.0 post hoc LSD), respectively. We used T-test because of our goal for comparing just MCAO and intact groups. Data were expressed as mean ± SEM. P<0.05 was considered significant.

Results
The effect of olive oil on neurological deficits scores
The results of the present research displayed that neurological deficits significantly improved in rats which received pre-treatment of olive oil with dose of 0.75 ml/kg/day (8.2±10.7, P=0.00) in comparison with the control group (16.32±10.7). Furthermore, the significant reduction of neurological score was observed in the sham group in comparison with the control group (2.32±10.7, P= 0.004) (Fig. 1).

The effect of olive oil on infarct volume
The present results indicated that the pre-treatment of dose of 0.75 ml/kg/day olive oil significantly reduced infarct volume in total of brain hemisphere (119.15±19.2 mm$^3$, P=0.001) in comparison with control group (213.78±8.3 mm$^3$). Furthermore, olive oil attenuated infarct volume in cortex (49.32±19.2 mm$^3$, P=0.03) and striatum (19.33±6.05 mm$^3$, P=0.008) areas in dose of 0.75 ml/kg/day when compared with the control group (107.44±6.4 and 67.57±3.2 mm$^3$). The doses 0.25 and 0.5 ml/kg/day of olive oil had no effect on infarction volume (Fig. 2 and 3).

Effects of pre-treatment with olive oil on NF-κB expression
Analysis of western blotting showed that the protein level of NF-κB was reduced in dose of 0.75 ml/kg/day of olive oil in the cortex and striatum compared with the control (intact) group (P=0.00 and P=0.03, respectively), while there was no significant difference in doses of 0.25 and 0.50 ml/kg/day. Therefore, it seems that the pre-treatment of olive oil could in dose-dependent manner cause down-regulation of NF-KB expression (Fig. 4).

Effects of pre-treatment with olive oil on TNFR1 expression
The assessment of TNFR1 protein level was carried out by western blotting technique. The results demonstrated that the olive oil in dose of 0.75 ml/kg/day in the cortex and striatum attenuated...
TNFR1 expression compared with the control (intact) group ($P = 0.01$ and $P = 0.00$, respectively). The significant difference in lower doses of olive oil was not seen. It is worthy of mentioning that the MCAO surgery led to the up-regulation of TNFR1 protein; this difference was significant in the cortex and striatum areas of the brain of the rat ($P=0.03$ and $P=0.03$, respectively). Hence, it can be reported that the pre-treatment of olive oil could cause the reduction of TNFR1 protein level in a dose-dependent response (Fig. 5).

**Discussion**

The results of the present study indicate that the oral administration of the olive oil, in addition to decreasing infarct volume, improves the neuro-motor impairments induced by the cerebral ischemia. It is worthy of mentioning that dose of 0.75 ml/kg/day of olive oil in cerebral ischemia decreased the infarction volume in striatum and cortex areas. The reports from the previous studies also give credence to the present study; they showed that virgin olive oil...
induces ischemic tolerance through reducing the infract volume, blood-brain barrier permeability, neurological deficits, cerebral edema and up regulating of the antioxidant enzymes activity (Mohagheghi et al., 2010). In the early stages of cerebral ischemia, the increase in free radical production plays a significant and undeniable role in the formation of edema and the development of cerebral ischemia-related infarction (Chen et al., 2011). Furthermore, the reperfusion-resulted injures are caused by the release of the inflammatory factors and free radicals in the damaged tissue (Huang et al., 2010). In addition, free radicals, as oxidants in the body, will react with saturated fat, leading DNA damage and neuronal death through peroxide production (Pradillo et al., 2006). Antioxidants are one of the therapeutic interventions that can attenuate the complications of cerebral ischemia. Meanwhile, olive oil, aside from having unsaturated fatty acids, contains phenolic compounds, too. Phenolic compounds of olive oil is defined as scavengers of free radicals and regulators of the antioxidant system (Visioli et al., 2002). According to the results of the past and the present studies, it can be said that the

<table>
<thead>
<tr>
<th>Brain Areas</th>
<th>Factors</th>
<th>Experimental Groups</th>
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<tr>
<td></td>
<td></td>
<td>Sham (Intact)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control (MCAO)</td>
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<tr>
<td></td>
<td></td>
<td>Olive Oil (0.25 ml/kg)</td>
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<td></td>
<td>Olive Oil (0.5 ml/kg)</td>
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<td></td>
<td></td>
<td>Olive Oil (0.75 ml/kg)</td>
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<tr>
<td>Cortex</td>
<td>TNFR1 (55 KD)</td>
<td>Western blot and its densitometric analysis of TNFR1 protein 24 h after the last administration of olive oil was done. All the data was expressed as the mean ± SEM and were normalized on the basis of β-actin levels (n=5). P&lt;0.05, compared with control (intact) group (Two-way ANOVA test).</td>
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<tr>
<td>Cortex</td>
<td>β-actin (42 KD)</td>
<td></td>
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<tr>
<td>Striatum</td>
<td>TNFR1 (55 KD)</td>
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<tr>
<td>Striatum</td>
<td>β-actin (42 KD)</td>
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**Fig.5.** The effect of various doses of olive oil (0.25, 0.5 and 0.75 ml/kg/day) on TNFR1 protein expression in cortex and striatum areas of hemisphere. Western blot and its densitometric analysis of TNFR1 protein 24 h after the last administration of olive oil was done. All the data was expressed as the mean ± SEM and were normalized on the basis of β-actin levels (n=5). P<0.05, compared with control (intact) group (Two-way ANOVA test).
neuroprotective effects of olive oil in cerebral ischemia are related to its antioxidant property (Sarshoori et al., 2014). Probably, olive oil with outstanding properties can prevent cell death caused by oxidative stress and free radicals as well (Esmailzadeh et al., 2004). The previous studies have also demonstrated that the excessive production of free radicals plays a crucial role in the excitotoxicity and triggers neuronal cell death. Likewise, antioxidant compounds are capable of ameliorating these kinds of detrimental ischemic damages (Bgdeli et al., 2008). Hydroxyl-tyrosol, another important component of olive oil, exerts its antioxidant activity in the brain through selective interaction with signaling cascades like tyrosine kinase, PKC, ptdins3-kinase and MAP kinase. These pathways regulate cell survival during oxidative stress (Shih et al., 2015). In this respect, Zamani et al., demonstrated that oral administration of olive oil for a week before the treatment and its reuse a week after the induction of global ischemia in mice, can reduce cell death in the temporal cortex and hippocampal CA1 areas and also improve memory impairment (Zamani et al., 2013). Hence, the infarct volume which occurs as a leading indicator of the cell death in cerebral ischemia, is probably due to the impact of useful compounds of olive oil on neuronal survival.

As it was mentioned, in the present study, pre-treatment with olive oil in cerebral ischemia greatly improved the neuro-motor deficits. It can be declared that the considerable improvement of neurological defects by olive oil (due to the antioxidant properties) may be attributed to the diminished cerebral infarction. The results of the present study are fully consistent with recent study too (Sarshoori et al., 2014).

It should also be stated that the present study displayed the decreasing effect of olive oil on NF-κB and TNFR1 protein level; this result highlights the inhibitory effect of olive oil on the destructive inflammatory process. TNFR1 expression in cortex and striatum areas was significantly decreased by dose of 0.75 ml/kg/day of olive oil. Furthermore, dose of 0.75 ml/kg/day of olive dose attenuated protein level of NF-κB significantly. The decrease in NF-κB protein level was observed in the cortex and striatum, too. This remarkable effect of olive oil in reducing the inflammatory factors protein level of cerebral areas, however, should not come as a surprise. This conclusion can be related to the fact that NF-κB factor be protein level in different areas of the rat brain such as amygdala, cortex, cerebellum, hippocampus, hypothalamus and olfactory lobes; it also has a very high level of activity in these areas (Shih et al., 2015). In order to evaluate the efficacy of olive oil in the inflammatory process, factors and complexes, which initiate the inflammation, are considered as the suitable candidates. Thus, the assessment of TNFR1 protein level expression as the initiator in signaling pathway of inflammation and NF-κB protein complex as the initiator of many cytokines and pre-inflammatory factors protein level expression, can be of great help for understanding the role of olive oil in the inflammatory pathways. Considerable evidence, however, has confirmed the increase of NF-κB activity during the cerebral ischemia and its participation in cell death caused by cerebral ischemia (Nurmi et al., 2004; Ridder and Schwaninger, 2009). Unlike the common role of NF-κB as an anti-apoptotic factor, this compound participates in cell death induced by cerebral ischemia. In fact, the NF-κB transcription factor is a key regulator in the inflammatory responses which can accelerate cell death. Therefore, the pathway which activates NF-κB in cerebral ischemia is determined from two perspectives: inflammation and apoptosis (Shih et al., 2015). With regard to the relationship between the activity of NF-κB and cell death, it can be mentioned that this nuclear factor can transcript numerous pre-inflammatory genes and augment their protein expression. The activity of these pre-inflammatory genes is known as the triggers of cell death in cerebral ischemia (Andreadou et al., 2006). The researchers’ findings indicated that phenolic compounds of olive oil prevent the blood-brain barrier integrity by suppressing free radicals, NF-κB activity and matrix metalloproteinase-9 expression (Sharma et al., 2000).

Once again the anti-inflammatory properties of olive oil with reduced expression of TNFR1 (main receptor responsible for TNF-α-induced cell death) was confirmed. The decreasing effect of olive oil on the protein level expression of TNFR1 was observed with dose 0.75 ml/kg/day. The involvement and up-regulation of TNF-α and TNFR1 have been indicated in cerebral ischemia (Botchkina et al., 1997; Castillo et al., 2010) as, high protein level of TNF-α was observed in the present study.
TNF1 has the most important role in the biological activity of TNF-α (Chen and Goeddel, 2002). TNF1 is actively involved in activating NF-κB, regulating inflammation and the apoptosis (Chen and Goeddel, 2002). TNF1 in particular has the paramount importance in triggering the inflammatory cascade. Moreover, binding TNF-α to TNF1 can lead to the apoptotic pathway through caspase-8 or as an activator of NF-κB. Possibly, there is a direct relation between NF-κB/TNF1 signaling pathway and neuronal death that occurs as infarction in cerebral ischemia.

In this regard, given the present results, it is safe to say that the pre-treatment of olive oil in cerebral ischemia displayed its neuroprotective role. This compound via decreasing ischemic damages particularly by improving the neurologic deficits and attenuating the infarction volume, opens obvious approaches for ameliorating the damages in individuals which are vulnerable to the cerebral ischemia. According to the findings of the present study, it can be supposed that the reduction in olive oil-induced ischemic damages are partially associated with the inhibition of inflammatory factors expression such as NF-κB and TNF1. Further researches, however, are warranted to cast some more light on the mechanisms which are involved in this regard.

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Conflict of interest
There is no conflict of interest.

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