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Oxidative Stress Induced by Permanent Cerebral Hypoperfusion Improved by Red Grape Seed Extract in Rats

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Introduction: This study aimed to evaluate the effect of chronic oral administration of red grape seed extract (GSE) on brain oxidative stress induced after permanent bilateral common carotid arteries occlusion (BCCAO) in male adult rats.

Methods: Thirty-two adult male Wistar rats (220±20g). The rats were divided randomly into four groups of 8 in each: 1) Sham+Veh; 2) Sham+GSE; 3) Isch+Veh; 4) Isch+GSE. In order to make animal model of permanent cerebral hypoperfusion/ischemia, both carotid arteries were ligated upper and lower and cut bilaterally. The animals in the treatment groups received a dose of 100 mg/kg GSE daily, by oral gavage (PO) for 4 weeks. Malondialdehyde (MDA) levels, Glutathione peroxidase (GPx) activity, and total thiol (–SH) groups were measured in homogenate of cerebral hippocampus and cortex.

Results: MDA elevated significantly in rats' hippocampus and cortex after chronic cerebral hypoperfusion/ischemia when compared with sham rats ($P<0.05$ and $P<0.001$ respectively). Biochemical examinations revealed that GSE reversed the increased level of brain tissue malondialdehyde (MDA), and attenuated decreased activity of glutathione peroxidase (GPx) in BCCAO rats.

Conclusion: These findings suggest that GSE exhibits therapeutic potential for oxidative stress induced by cerebral hypoperfusion/ischemia, which is most likely related, at least in part, to its antioxidative and free radical scavenging actions.

Keywords: Cerebral ischemia, Oxidative Stress, Grape Seed Extract, Rat.

Introduction

It has been widely accepted that chronic cerebral hypoperfusion induces oxidative stress damage in neural tissues and cells, at least partially due to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Aliev et al., 2003; Nejad et al., 2015).

Studies have shown that a chronic mild hypoperfusion induced by permanent bilateral common carotid arteries occlusion (BCCAO) causes learning and memory deficits and that this progressive cognitive deficit parallels the progress of neuronal damage (Ni et al., 1994; Ashabi et al., 2015). Global cerebral ischemia in rodents is an established model in experimental research on cerebral ischemia, which is characterized morphologically by a selective neuronal damage in the hippocampus, striatum and cortex.

Using this model, many studies have examined the pathophysiology of ischemic neuronal damage. Based upon these results, it has been investigated whether substances which interact with the pathophysiological processes reduce the ischemic neuronal damage (Block, 1999; Ashabi et al., 2015). It is well known that ischemia causes neuronal necrosis in selectively vulnerable sectors of the hippocampus, because the hippocampus involves the spatial navigation, learning and memory. Selective deficits in these areas may arise from ischemic brain damage (Auer et al., 1989).Oxygen free radicals or oxidants are thought to be involved in acute central nervous system injury induced by cerebral ischemia (Chan, 1996; Mansouri et al., 2014). Although ischemia disrupts cerebral blood flow (CBF), it can lead to brain injury from influx of neutrophils and

increases ROS, cerebral edema, and hemorrhage (Harukuni & Bhardwaj, 2006). Permanent BCCAO in rats has been used as an animal model of chronic cerebral hypoperfusion ischemia phenolic compounds, such as catechin, epicatechin, as dimeric and tetramer proanthocyanidin (Bagchi et al., 2002). The beneficial effects of grape seed polyphenols are due to their free radical scavenging capability, but the antioxidant activity of grape seed and grape juice polyphenols is superior to other well-known antioxidants, such as vitamin C, vitamin E, and beta-carotene (Shinno et al., 1997; Andres-Lacueva et al., 2005; Shukitt-Hale et al., 2006). Antioxidants are potential candidates for prevention or treatment of disorders involving oxidative stress (Pfleger et al., 2010). Antioxidant activities might be a major contributory factor to the role of grape seed polyphenols in ischemic injuries and brain inflammation (Wang et al., 2009; Karaaslan et al., 2010). This study aimed to investigate the effects of chronic oral administration of GSE on brain tissue oxidative stress during cerebral hypoperfusion/ ischemia in rat model of permanent BCCAO.

Materials and Methods

Animals and Experimental Procedure

Thirty-two adult male Wistar rats (220±20g) obtained from animal laboratory of Ahvaz Jundishapur University of Medical Sciences (AJUMS) were used in this study. They were housed individually in standard cages under controlled room temperature (20±2°C), humidity (50-55%), 12:12h light/dark cycle and free access to food and water ad libitum. All experiments were monitored by the Local Ethics Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals. Ten days after receiving them and accommodating with laboratory conditions, the rats were randomly divided into four groups of 8: 1) Isch+GSE; their common carotid arteries were cut bilaterally and received 28 days hydroalcoholic GSE (100mg/kg, by oral gavages); 2) Isch+Veh, their carotid arteries were cut bilaterally and received 28 days the same volume of normal saline as GSE vehicle; 3) Sham+GSE, surgeries were done but their carotid arteries remained intact and received 28 days GSE (100mg/kg, gavages); 4) Sham+Veh, their common carotid arteries remained intact and received 28 days GSE vehicle (Hwang *et al.*, 2004). This dose of GSE was selected as the effective dose based on our previous dose response experiments carried out in our laboratory (Badavi *et al.*, 2008; Farbood *et al.*, 2009).

Surgery

In order to make the permanent cerebral hypoperfusion/ischemia in the rats, we used Cechetti's method (2010) with little modification. Briefly, in anesthetized rats with ketamine/xylazine (50/5mg/kg, i.p) both common carotid arteries concomitantly occluded by upper and lower ligatures (2-0 silk suture) and then cut bilaterally. In sham-operated groups (Sham+Veh and Sham+GSE), the same surgical procedure was done but carotid arteries remained intact (Cechetti *et al.*, 2010).

GSE preparation

Grape fruits (*vitis vinifera Lin.*), as large clusters with red berries, were bought from Qazvin province (northwest of Iran) red grape gardens. Seeds were removed from the grapes, air dried (in shade) for one week and milled to fine powder (a particle size of <0.4mm). The grape seed powder was macerated in 70% ethanol (25% w/v) for 72h at room temperature and was stirred three times a day. The ethanol extract evaporated to remove ethanol, and grape seed extract was obtained as a lyophilized powder (yield: 25-30%). GSE extract was dissolved in normal saline and animals in treated groups received a daily dosage of 100 mg/kg by oral gavage (PO) for 4 weeks (Farbood *et al.*, 2009; Sarkaki *et al.*, 2012).

Estimation of biochemical parameters

At the end of the experiments, animals were sacrificed by decapitation under deep anesthetization with higher doses of ketamine/xylazine and brains were removed by fast craniotomy and rinsed with ice-cold isotonic normal saline. The hippocampus and cortex portions of the removed brain were frozen in liquid nitrogen and stored at -80ºC to the point of brain tissue homogenate preparation. Brain tissue samples were then homogenized with ice cold 1. 5% KCl in a volume 10 times the weight of the tissue. The homogenate was centrifuged, aliquots of supernatant were separated and used for biochemical estimation.

Malondialdehyde measurement

Malondialdehyde (MDA) levels, an index of lipid peroxidation, produced by free radicals were measured. MDA reacts with thiobarbituric acid to produce a red colored complex that has peak absorbance at 532 nm. Briefly, 3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) were added to 0.5 ml of homogenate in a centrifuge tube and the mixture was heated for 45 minutes in a boiling water bath. After cooling, 4 ml n-butanol was added to the mixture and vortex-mixed for 1 min followed by centrifugation at 2000 rpm for 20 min. The colored layer was transferred to a fresh tube and its absorbance was measured at 532 nm. MDA levels were determined using 1, 1,3,3- tetramethoxypropane as standard. The standard curve of MDA was constructed over the concentration range of 0–20 l M (Ohkawa *et al.*, 1979; Naghizadeh *et al.*, 2010).

Estimation of GPx activity

Glutathione peroxidase (GPx) activity was assayed using a commercial kit obtained from Randox Laboratories (RANSEL) (Candelario-Jalil *et al.*, 2001).

Total thiol (–SH) groups

Total SH groups were measured using DTNB (5, 5´-dithiobis-2-nitrobenzoic acid) as the reagent (Ellman, 1959; Naghizadeh *et al.*, 2010). This reagent reacts with the SH groups to produce a yellow colored complex which has a peak absorbance at 412 nm. Briefly, 1 ml Tris–EDTA buffer (pH 8.6) was added to 50 µl brain tissues homogenate in 2 ml cuvettes and absorbance was read at 412 nm against Tris–EDTA buffer alone (A1). Then, 20 µl DTNB reagents (10 mM in methanol) was added to the mixture and after 15 minutes (stored in laboratory temperature), the sample absorbance was read again (A2).

The absorbance of DTNB reagent was also read as a blank (B). Total thiol concentration (mM) was calculated using the following equation: Total thiol concentration (mM) = (A2- A1-B) x 1.07/0.05* 13.6.

Statistical Analysis

Data were expressed as mean±S.E.M. Oxidative stress in cerebral cortex and hippocampus regions were analyzed by one-way ANOVA followed by LSD post hoc tests. The statistical level of significance was considered at $p<0.05$.

Results

Biochemical parameters

Figure 1 represents **(A)** the Glutathione peroxides (GPx), **(B)** Malondialdehyde (MDA) and **(C)** total thiol in brain cortex tissue of ischemic (Isch+Veh) and sham operated (Sham+Veh) groups after 28 days treatment with GSE or vehicle. GPx was lowered significantly (#P<0.05) in Isch+Veh and Isch+GSE in comparison with Sham+Veh and Sham+GSE respectively. Oral administration of GSE for 28 days increased the GPx significantly (***P<0.001) in ischemic and sham groups compared with to non-treated groups (Isch+Veh and Sham+Veh). MDA increased significantly (###P<0.001) after ischemia induced by permanent 2CCAO. Oral administration of GSE for 28 days decreased it significantly (***P<0.001) in ischemic but not in sham group. The total thiol in brain cortex region did not change significantly, neither in treated nor in non-treated sham or ischemic groups. Figure 2 represents **(A)** the Glutathione peroxides (GPx), **(B)** Malondialdehyde (MDA) and **(C)** total thiol in hippocampus region of brain tissue of ischemic and sham groups after treatment with GSE or vehicle for 28 days. GPX decreased significantly after permanent BCCAO (###P<0.001for Isch+Veh vs. Sham+Veh) and oral administration of GSE for 28 days increased it significantly in ischemic but not in sham group (*P<0.05 for Isch+GSE vs. Isch+Veh). MDA increased significantly after permanent BCCAO (###P<0.001 for Isch+Veh vs. Sham+Veh). Oral administration of GSE for 28 days decreased it significantly in both sham and ischemic groups (***P<0.001for Sham+GSE and Isch+GSE vs. Sham+Veh and Isch+Veh respectively). Total thiol increased significantly after ischemia induced by permanent BCCAO (#P<0.05 for Isch+Veh vs. Sham+Veh and and ###P<0.001 for Isch+GSE vs. Sham+GSE respectively). Treatment with GSE could not significantly reverse total thiol in either sham or the ischemic group.

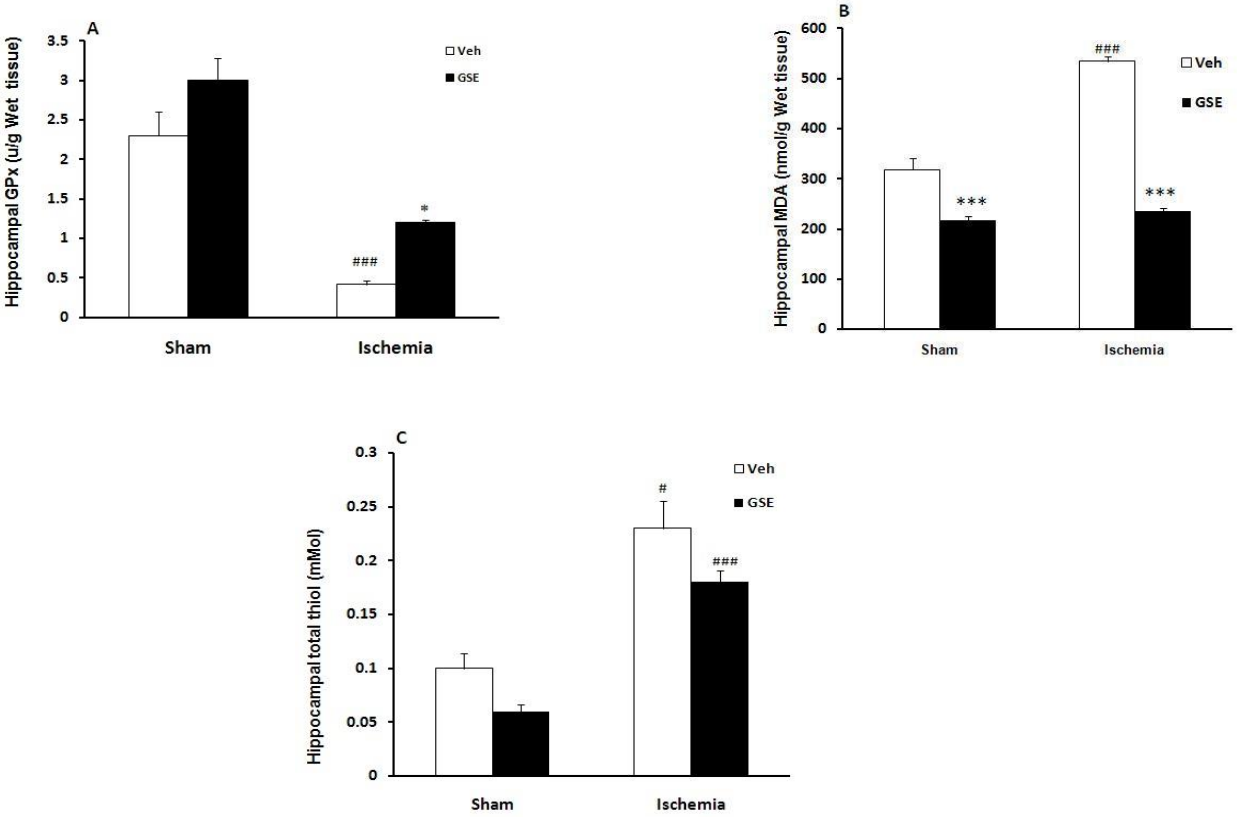

Figure 1: Mean± S.E.M. of (A) glutathion peroxidase (GPx), (B) malondialdehyde (MDA) and (C) total thiol measured in cortex region of brain tissue in Sham+Veh, Sham+GSE, Isch+Veh and Isch+GSE groups. One -way ANOVA, followed by LSD post hoc test, n=6, ***P<0.001 for groups treated with GSE for 28 days vs. groups treated with Vehicle for same time. #P<0.05 for GPx in Isch+GSE vs. sham+GSE and ###P<0.001 for MDA in Isch+Veh vs. Sham+Veh.

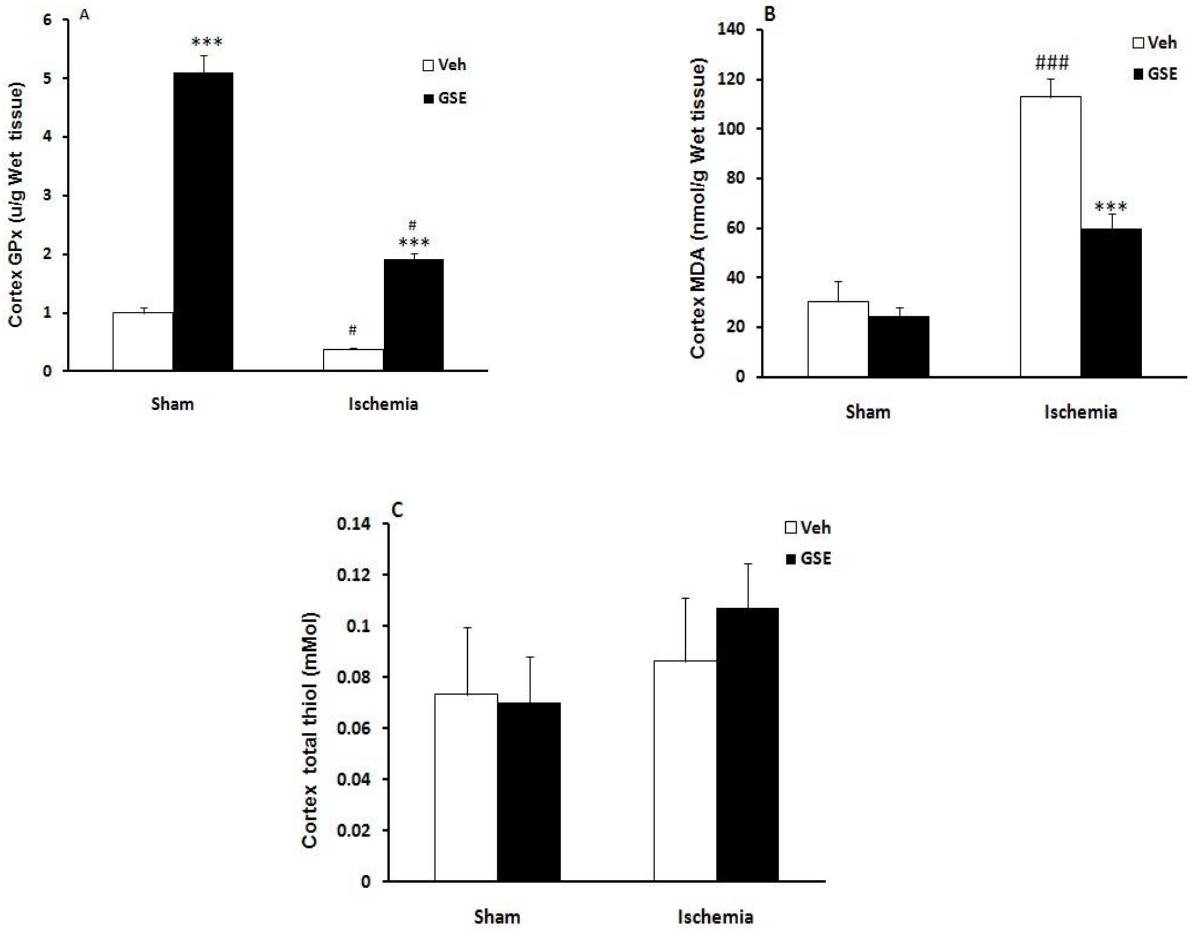

Figure 2: Mean± S.E.M. of (A) glutathion peroxidase (GPx), (B) malondialdehyde (MDA) and (C) total thiol measured in hippocampus region of brain tissue in Sham+Veh, Sham+GSE, Isch+Veh and Isch+GSE groups. One-way ANOVA, followed by LSD post hoc test, n=6, *P<0.05 and ***P<0.001 in Sham+GSE vs. Sham+Veh, #P<0.05 and ###P<0.00 in Isch+Veh vs. Sham+Veh for GPx and MDA.

Discussion

Our findings showed that chronic treatment of sham operated and ischemic rats with grape seed extract (GSE) could increase GPx, decrease MDA in brain tissues but couldn't reverse total thiol. Cerebral ischemic stroke is a neurological disease where neuronal cell death is caused by a serial pathophysiological events, the so-called 'ischemic cascade' like energy failure, excitotoxicity, oxidative stress, inflammation, apoptosis etc. (Farkas *et al.*, 2007; Ashabi *et al.*, 2015). Oxygen radicals play a crucial role in brain injury. Grape seed extract is a potent anti-oxidant. Treatment with grape seed extract suppresses lipid peroxidation and reduces hypoxic ischemic brain injury in rats (Feng *et al.*, 2005; Sarkaki *et al.*, 2014).

Hippocampus is very sensitive to ischemia and the some of its subdivisions are characterized by a low capillary density as compared with the neighboring other subdivisions (Cavaglia *et al.*, 2001). Oxidative stress is believed to be involved in the damaging mechanism of excitotoxic insult. GSE, as a potent antioxidant with inhibitory capacity of free radicals, acts as a neuroprotectant (Shi *et al.*, 2003; Farkas *et al.*, 2007). It has been reported that GSE with polyphenols and proanthocyanidins (PA) acts directly as antioxidants by scavenging reactive oxygen (Frei & Higdon, 2003; Scalbert *et al.*, 2005; Mansouri *et al.*, 2014). It reduces the elevated 8-iso prostaglandin F2α by hypoxia/ischemia (HI) and also reduces the elevated pro apoptotic protein c-jun in cerebral cortex by HI (Deshane *et al.*, 2004). Oral administration of GSE reduced MDA

significantly in cortex and hippocampus regions of BCCAO rats (figures 1 and 2). Hippocampus and striatum are susceptible to damage induced by oxidative stress and free radicals (Lau *et al.*, 2005). It has been revealed that ROS production and oxidative stress induction are known as harmful agents in injured brain. These factors lead to oxidized lipids and proteins in central nervous system, followed by neuronal damage or death (O'Donnell *et al.*, 2000; Lau *et al.*, 2005). Studies showed that lipid peroxidation (LPO) produces various molecules such as aldehydes and MDA (Niki, 2009). LPO is a reaction of free radicals with cell membrane lipids, proteins, enzymes and also with nucleic acids (Pratico, 2008), which produce unsaturated fatty acids (Esposito *et al.*, 2002). Therefore LPO could change structural, chemical and physical properties of cell membrane and contribute to reducing its performance in neurodegenerative diseases (Sultana *et al.*, 2006). Oral administration of GSE enhanced the antioxidant status and decreased the elevated free radical-induced by lipid peroxidation to normal values in the rat central nervous system (Balu *et al.*, 2005; Raafat *et al.*, 2010).

In this experiment glutathione peroxidase (GPx) activity reduced significantly in ischemic brain regions. Total thiol increased significantly in the hippocampus but not in cortex region of rats' brain with BCCAO (figures 1 and 2). Glutathione is a tripeptide that is a reductant for glutathione peroxidase (Wang *et al.*, 2009).

During ischemia, mitochondrial glutathione is released from the mitochondrion into the cytosol forming a unique pool that leaks out to the interstitial space (Rigobello & Bindoli, 1993) and reperfusion causes a large release of total glutathione, particularly from cytosol (Lu, 1998). In this experiment, GPx (but not thiol) increased in both cortex and hippocampus regions of sham-operated (sham) and BCCAO (ischemic) groups after 28 days GSE consumption (figures 1 and 2). Glutathione is synthesized enzymatically by γ-glutamylcysteine synthetase (γ_GCS) and glutathione synthetase, with the former being the rate-limiting enzyme (Lu, 1998). This could be explained by the expression of γ -glutamyl cysteine synthetase, the rate-limiting enzyme for GSH synthesis in flavonoids (Balu *et al.*, 2005). These previous results confirm our finding about the effect of GSE consumption on GPx and total thiol in the current investigation. As shown in figures 1 and 2 permanent hypoperfusion/ischemia caused lipid peroxidation in brain tissues resulted in MDA elevation in both cortex and hippocampus. Treated ischemic rats with GSE chronically inhibited lipid peroxidation significantly and lowered MDA level in mentioned brain tissues of sham and ischemic groups. Oxygen radicals may induce lipid peroxidation by the means of the reaction with lipid structures. Intensity of lipoperoxidation was measured by detection of substances giving positive reaction with thiobarbituric acid (TBA) in homogenates of brain tissues (Halcak *et al.*, 1998). Elevated extracellular glutamate levels during cerebral ischemia causes malondialdehyde production (MDA), a common index of lipid peroxidation in rat brain tissues. Elevation of extracellular glutamate levels in brain induces neurotoxicity and damages the brain neurons. These results suggest that excitotoxicity induces oxidative stress in rat brain, as evidenced by the glutamate-induced increase in malondialdehyde production (Yang *et al.*, 1998). GSE decreased MDA with effects on the lipid peroxidation process. Several lines of studies suggested that grape seed proanthocyanidin extract (GSPE) administration as a plant antioxidant markedly suppressed lipid peroxidation and free radicals as well as the activation of iNOS, and calpainII (Li *et al.*, 2012; Zhang & Hu, 2012). In a study to assess the oxidative stress in acutely and chronically exercised rats, it was shown that GSE supplementation caused an increase in antioxidant enzyme activities and prevented exercise-induced oxidative stress by preventing lipid peroxidation and increasing antioxidant enzyme activities (Belviranli *et al.*, 2012).

It has been suggested that GSE is a useful herbal remedy, especially for controlling oxidative damages and is considered as a potent protective agent against lipid peroxidation (El-Ashmawy *et al.*, 2010). On the other hand, grape pomace (grape seed extract and grape peel powder) supplementation is considered to activate the antioxidant enzyme system and prevents damage with hypercholesterolemia by lowering the formation of lipid peroxide (Choi *et al.*, 2010). Another investigation suggested intake of GSE may be a feasible therapeutic strategy for prevention of oxidative stress (Suwannaphet *et al.*, 2010). The results of another study suggested that the grape seed extract enhanced the antioxidant defence against reactive oxygen species produced under hyperglycaemic conditions (Chis *et al.*, 2009). It has been found that even Shahani black grape has also potent antioxidant activity specially on lipid peroxidation and has beneficial effects on human health and help to prevent diseases which are caused by free radicals (Yassa *et al.*, 2008).

Results of studies designed to evaluate grape seed extract (GSE) and vitamin E supplements on lipid peroxidation, on antioxidant systems and peripheral blood lymphocytes in rats exposed to x-rays, indicate that GSE enhanced the antioxidant status and decreased the incidence of free radical-induced lipid peroxidation in blood samples. The antioxidant effect of GSE given to animals was more effective than vitamin E administered before whole-body irradiation in rats (Enginar *et al.*, 2007).

Conclusion

Our results showed that oral administration of GSE improved oxidative stress which occurs during brain injury induced by BCCAO. Therefore, it may have the potential to be an effective free radical scavenger for preventing neurodegenerative disorders due to cerebral ischemia.

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Conflict of Interest

There is no conflict of interest to be declared.

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