

Full Length Research Paper

Resveratrol alleviates oxidative stress and inflammation in the hippocampus of rats subjected to global cerebral ischemia/reperfusion: Comparison with vitamin E

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Resveratrol is a dietary polyphenol present in nuts and dried fruits. It is a potent antioxidant and anti-inflammatory agent; these properties led researchers to investigate its protective effects in several animal models of neurological disease, particularly those in which inflammation and oxidative stress may play a role in their pathogenesis. Thus it seemed interesting to study the possible neuro-protective effect of resveratrol and vitamin E in cerebral ischemia/reperfusion (IR)-induced hippocampal damage in brains of rats. Rats were divided into four groups (n=10); groups 1 and 2 were given 0.5 ml (1% tween 80, po) while groups 3 and 4 were treated with vitamin E (100 mg/kg, po) and resveratrol (10 mg/kg po), respectively. All treatments were administered for 14 days and on the 15th day of the experiment, animals were anaesthetized with thiopental (50 mg/kg, ip) and IR was induced by occlusion of both carotid arteries for 60 min, followed by reperfusion for another 60 min in all groups except for the sham-operated group. At the end of reperfusion, the rats were sacrificed by decapitation and brains removed, hippocampi isolated and homogenized in ice cold saline for estimation of lactate dehydrogenase activity, oxidative stress markers (thiobarbituric acid reactive substances, total nitrate/nitrite and total antioxidant capacity) and inflammatory biomarkers (myeloperoxidase activity, tumor necrosis factor-alpha, interleukin-6 and interleukin-10 contents). Resveratrol and vitamin E protected against oxidative stress, lipid peroxidation and inflammation associated with IR injury.

Key words: Vitamin E, resveratrol, ischemia/reperfusion, stroke.

INTRODUCTION

Stroke is a rapidly developing signs of focal or global disturbance of cerebral function (Sudlow and Warlow, 1997). It usually occurs because a blood vessel bursts or is blocked by a clot. When blood flow to the brain is

interrupted an area of the brain is deprived of oxygen and other nutrients (Selman et al., 1997). Given that the brain requires an un-interrupted supply of blood, the longer the duration of cerebral ischemia the lower the chance of

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reversible injury. The area which is severely affected by the lack of cerebral blood flow is termed the "ischemic core" and cell death of both neurons and astrocytes is profound in this region (Kogure et al., 1992).

Independently from the mechanism responsible for the vessel occlusion, ischemia causes a cascade of events that eventually lead to neuronal damage and death (Fisher and Schaebitz, 2000). The decline of blood flow decreases high energy phosphates production. The energy failure results in membrane depolarization and abandoned release of excitatory aminoacids, such as glutamate, in the extracellular space and excitotoxicity. Glutamate acts on various types of receptors, namely, NMDA and AMPA, eventually causing calcium overload in neuronal cells. Neuronal nitric oxide synthase is also calcium dependent and produces nitric oxide (NO) that plays a crucial role in the course of cerebral ischemia by reacting with superoxide and generating highly reactive radical peroxynitrite (Moro et al., 2004). Secondary to ischemia pro-inflammatory genes are expressed and several inflammatory mediators are released. Currently, there is increasing evidence that inflammation plays a major role in the setting of cerebral ischemia (Tuttolomondo et al., 2012). Experimental studies have shown that inhibition of the inflammatory process has lead to decrease in the extent of injury, an aspect which has gained great importance in understanding and management of stroke (Adibhatla and Hatcher, 2008; Tănăsescu et al., 2008).

Although excitotoxicity typically leads to necrosis, it has been proposed that both apoptosis and necrosis processes are triggered in parallel during ischemia and that the specific conditions determine which one will predominate (Lee et al., 1999).

A wide variety of drugs, which interfere at various points in the ischemic cascade, so-called neuroprotective agents have been used in both experimental and clinical studies. Antioxidants have shown benefits in minimizing the extent of injury and neuronal loss in cerebral ischemia. Treatment of rats with the antioxidants such as vitamin E protects against reactive oxygen species and has decreased the extent of injury in different models of brain ischemia (Knuckey et al., 1995; Sekhon et al., 2003; Jatana et al., 2006).

Resveratrol, a polyphenol phytoalexin, abundantly found in grape skins, has been reported to have multiple physiological effects, including the prevention of lipid peroxidation in human LDL (Frankel et al., 1993), inhibition of a rachidonic acid metabolism (Shin et al., 1998), anti-oxidative (Gedik E1 Girgin et al., 2008; Mozaffarieh et al., 2008) and anti-inflammatory effects (Kubota et al., 2009; Csiszar, 2011). Several studies have shown that and resveratrol reduces the risk of atherosclerosis, renal and cardiovascular diseases (Hao and He, 2004; Raval et al., 2008). These protective actions of resveratrol in cerebral ischemia have so far been identified from studies *in vivo* and in cultured neurons (Ray et al., 1999; Raval et al., 2006; Han et al.,

2004). Although, early effects may be of little relevance to treating clinical stroke; they are necessary for understanding all the mechanisms of action of any potential therapeutic agent and may be beneficial if agents are being developed for prophylactic use. Therefore, the goal of the present study was to explore the protective effects of resveratrol in comparison to vitamin E on cerebral ischemia-reperfusion.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200 to 250 g were obtained from the animal facility of Faculty of Pharmacy, Cairo University. Rats were housed under controlled temperature ($25 \pm 2^\circ\text{C}$) and constant light cycle (12 h light/dark) and allowed free access to a standard rodent chow diet and water. The investigation complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University.

Chemicals

Vitamin E and resveratrol (trans-3,5,4'-Trihydroxystilbene, 99% (GC)) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other used chemicals were of analytical grade.

Experimental design

Rats were randomly divided into five groups, 8 animals each. Group 1: served as a sham operated group in which a midline incision was made and both carotid arteries were exposed for 2 h without occlusion. Group 2: served as ischemic control (I/R) group. Groups 3 and 4: received vitamin E (100 mg/kg) or resveratrol (10 mg/kg), respectively for 14 days and on the 15th day I/R was induced. All drugs were freshly prepared daily in saline, administered orally.

Induction of cerebral ischemia/reperfusion

Rats were anaesthetized with (50 mg/kg thiopental, ip). Hair around the neck was shaved, a midline incision was made and both carotid arteries were exposed. Each carotid artery was freed from its adventitial sheath and vagus nerve (Ulrich et al., 1998). Both arteries were then occluded for 60 min using bull dog clip. Then the carotids were de-clamped and reperfusion was allowed for another 60 min (Seif-EI-Nasr and EI-Fattah, 1995). Temperature was kept constant during and after surgery using a heat lamp placed above the head of the animal in order to prevent cerebral hypothermia (Seif-EI-Nasr et al., 1992).

Biochemical measurements

Following I/R, animals were killed by decapitation, brains were removed and both hippocampi were isolated and homogenized in ice-cold NaCl (0.9%), using a homogenizer (HeidolphDiAx 900, Germany) to prepare 10% homogenate. The resultant homogenates were then used for determination of the following parameters.

Lactate dehydrogenase activity (LDH)

LDH was carried out according to the method described by Buhl and Jackson (1978) using Stanbio kit and expressed as U/g wet weight (Buhl and Jackson, 1978).

Lipid peroxidation products

Lipid peroxidation products were estimated by determination of the level of thiobarbituric acid reactive substances (TBARS) that were measured according to the assay of Buege and Aust (1978) and expressed as nmol/g wet weight.

Tissue total nitrate/nitrite (NO_x)

NO_x was determined spectrophotometrically at 540 nm using Griess reagent after reduction of nitrate to nitrite by vanadium trichloride (Miranda et al., 2001) and expressed as μmol/g wet weight.

Total antioxidant capacity (TAC)

TAC was assessed using rat total antioxidant capacity kit (BIODIAGNOSTIC, Egypt). The procedure of the used kit was performed according to the manufacturer's instructions and the results were expressed as pg/g wet weight.

Tumor necrosis factor-alpha (TNF-α)

TNF-α content was assessed using rat TNF-α ELISA kit (BD Biosciences, San Diego, USA). The procedure of the used kit was performed according to the manufacturer's instructions and the results were expressed as pg/g wet weight.

Interleukin-6 (IL-6)

IL-6 was assessed using rat IL-6 ELISA kit (BD Biosciences, San Diego, USA). The procedure of the used kit was performed according to the manufacturer's instructions and the results were expressed as pg/g wet weight.

Interleukin-10 (IL-10)

IL-10 was assessed using rat IL-10 ELISA kit (R&D, USA). The procedure of the used kit was performed according to the manufacturer's instructions and the results were expressed as pg/g wet weight.

Myeloperoxidase activity (MPO)

MPO was determined kinetically at 460 nm by measuring rate of H₂O₂-dependent oxidation of o-dianisidine that is catalyzed by MPO. One unit of MPO activity is defined as the amount of enzyme that degrades 1 μmol peroxide per min at 25°C (Bradley et al., 1982). MPO activity is expressed as mU/g wet weight.

Statistical analysis

All data obtained were presented as mean ± standard error of mean (SEM). Results were analyzed using one way analysis of variance

test (One-way ANOVA) followed by Student-Newman-Keuls multiple comparison test. Statistical analysis was performed using GraphPadInstat software (version 2.04). For all the statistical tests, the level of significance was fixed at p<0.05.

RESULTS

Effect on LDH activity

I/R was associated with elevation in LDH activity about 6 folds that is in the sham operated group. Pre-administration of vitamin E and resveratrol offered neuro-protection in our model of ischemia-reperfusion evident by a reduction in LDH activity to 49.41 and 31.22% of the I/R group, respectively (Figure 1).

Effect on oxidative stress biomarkers

Following induction of I/R, oxidative stress was manifested by a significant increase in lipid peroxidation manifested as 2 fold increase in TBARS content compared to sham operated group. Furthermore, I/R lowered the total antioxidant capacity to be 79.6% of that in sham operated group (Figures 2 and 3). Total nitrate/nitrite (NO_x) content was elevated almost 2 folds of that in the sham operated group. Pre-treatment with vitamin E or resveratrol reduced TBARS to be 77.64 and 78.84% of I/R group, respectively. Meanwhile, NO_x content was reduced only in vitamin E to be 62.76% of I/R group (Figure 4).

Effect on inflammatory biomarkers

Following induction of I/R inflammation was manifested by about 2-fold increase in TNF-α, IL-6 contents (Figures 5 and 6), together with a significant decrease in IL-10 content of 28.6% of the sham operated group (Figure 7). Pre-treatment with vitamin E or resveratrol reduced TNF-α to be 70 and 75% of the I/R group, respectively. Meanwhile, IL-6 was reduced to be 45.65 and 69.56% in vitamin E and resveratrol groups, respectively. On the other hand, both drugs elevated IL-10 to be 182.65 and 269.86% of the I/R group. I/R induced MPO activity to be 158.82% of the sham operated group and effect that was counteracted by pretreatment with vitamin E or resveratrol where MPO activity reached 74.07 and 59.25% in the aforementioned groups, respectively (Figure 8).

DISCUSSION

Interruption of cerebral blood flow leads to vascular leakage, inflammation, tissue injury, and necrosis (Wexler, 1970; Martins et al., 1980). Changes associated with ischemia include impairment of metabolism, energy

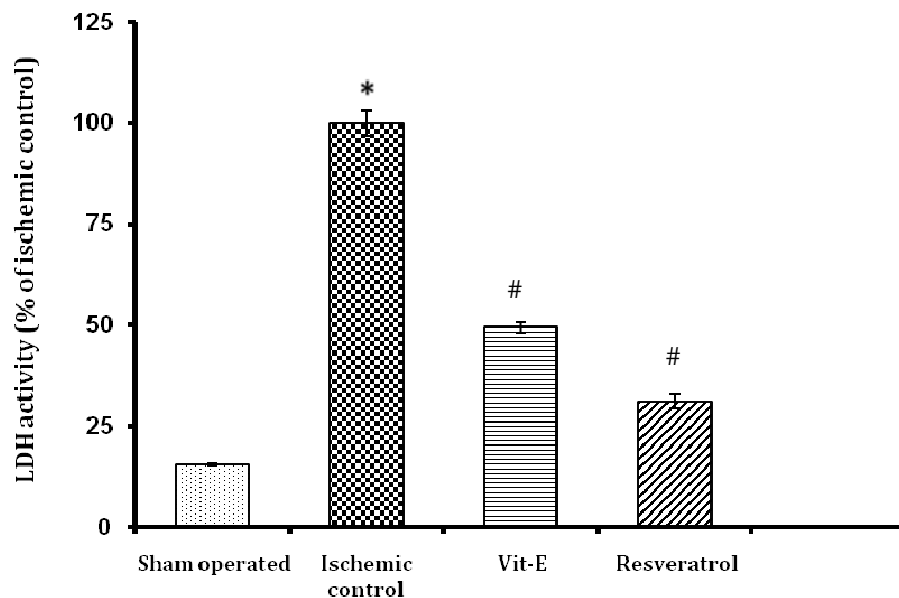


Figure 1. Effect of resveratrol and vitamin-E (Vit-E) on lactate dehydrogenase (LDH) activity in the hippocampus tissue in rats subjected to Ischemia/Reperfusion (I/R) injury. All drugs were administered for 14 consecutive days, and on the 15th day ischemia followed by reperfusion were performed. Data was expressed as means \pm S.E. and as percent of I/R control, $n = 8$. Statistical analysis was carried out by one-way ANOVA followed by the Tukey Kramer multiple comparisons test for comparison of means of different groups.

*Significantly different from sham operated group at $p < 0.05$.

#Significantly different from ischemic control group at $p < 0.05$.

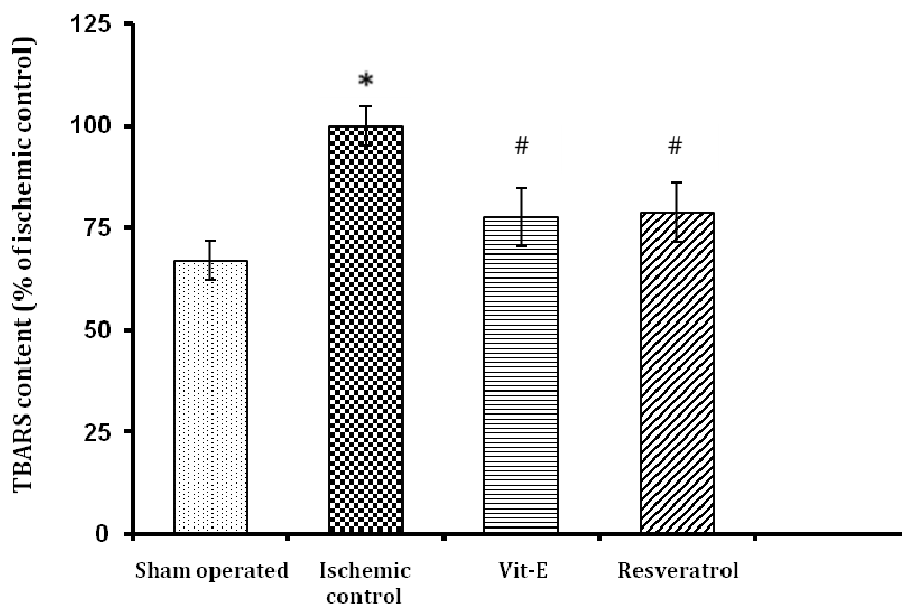


Figure 2. Effect of resveratrol and vitamin-E (Vit-E) on thiobarbituric acid reactive substances (TBARS) content in the hippocampus tissue of rats subjected to Ischemia/Reperfusion (I/R) injury. All drugs were administered for 14 consecutive days, and on the 15th day ischemia followed by reperfusion were performed. Data was expressed as means \pm S.E. and as % of I/R control, $n = 8$. Statistical analysis was carried out by one-way ANOVA followed by The Tukey Kramer multiple comparisons test for comparison of means of different groups.

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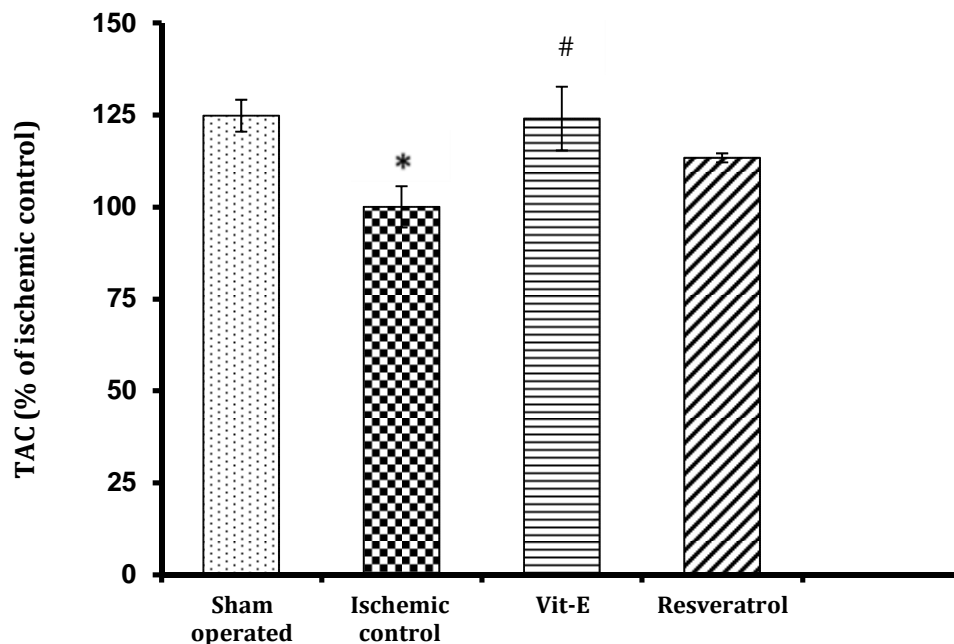


Figure 3. Effect of resveratrol and vitamin-E (Vit-E) on total antioxidant capacity (TAC) in the hippocampus tissue of rats subjected to Ischemia/Reperfusion (I/R) injury. All drugs were administered for 14 consecutive days, and on the 15th day ischemia followed by reperfusion were performed. Data was expressed as means \pm S.E. and as % of I/R control, $n = 8$. Statistical analysis was carried out by one-way ANOVA followed by The Tukey Kramer multiple comparisons test for comparison of means of different groups.

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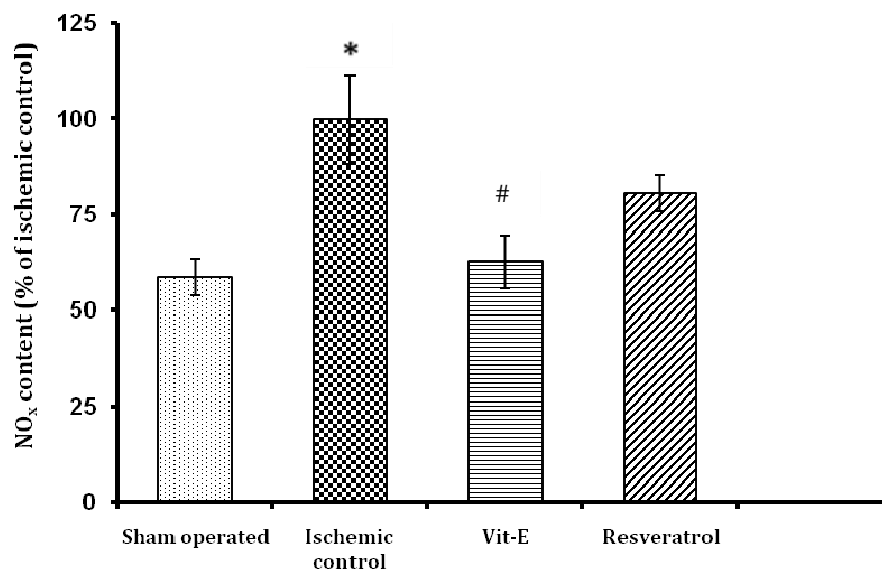


Figure 4. Effect of resveratrol and vitamin-E (Vit-E) on total nitrate/nitrite (NO_x) content in the hippocampus tissue of rats subjected to Ischemia/Reperfusion (I/R) injury. All drugs were administered for 14 consecutive days, and on the 15th day ischemia followed by reperfusion were performed. Data was expressed as means \pm S.E. and as % of I/R control, $n = 8$. Statistical analysis was carried out by one-way ANOVA followed by The Tukey Kramer multiple comparisons test for comparison of means of different groups.

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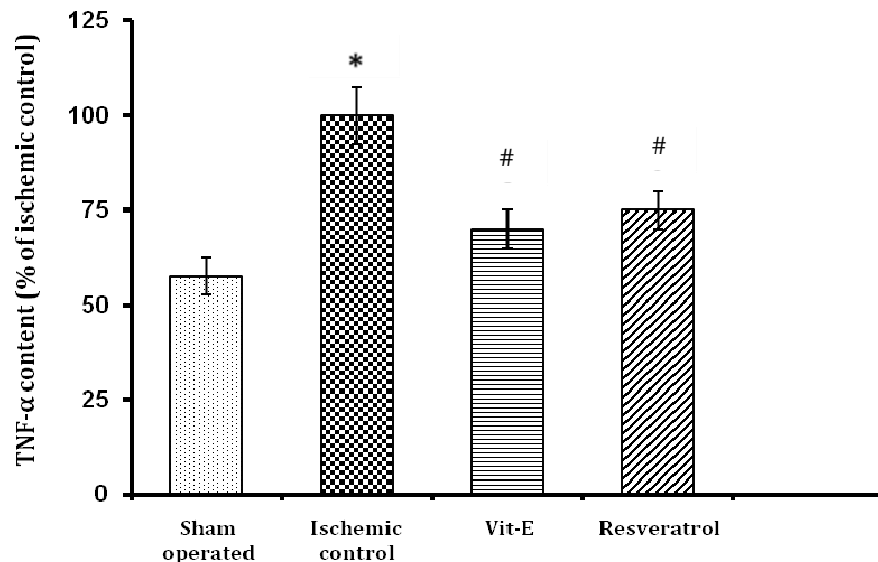


Figure 5. Effect of resveratrol and vitamin-E (Vit-E) on tumor necrosis factor-alpha (TNF- α) content in the hippocampus tissue of rats subjected to Ischemia/Reperfusion (I/R) injury. All drugs were administered for 14 consecutive days, and on the 15th day ischemia followed by reperfusion were performed. Data was expressed as means \pm S.E. and as % of I/R control, n = 8. Statistical analysis was carried out by one-way ANOVA followed by The Tukey Kramer multiple comparisons test for comparison of means of different groups.

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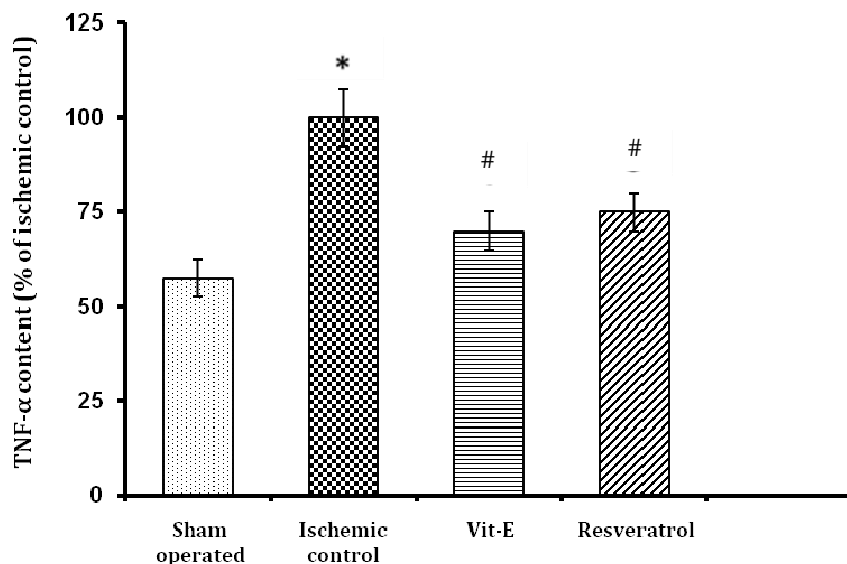


Figure 6. Effect of resveratrol and vitamin-E (Vit-E) on interleukin-6 (IL-6) content in the hippocampus tissue of rats subjected to Ischemia/Reperfusion (I/R) injury. All drugs were administered for 14 consecutive days, and on the 15th day ischemia followed by reperfusion were performed. Data was expressed as means \pm S.E. and as % of I/R control, n = 8. Statistical analysis was carried out by one-way ANOVA followed by The Tukey Kramer multiple comparisons test for comparison of means of different groups.

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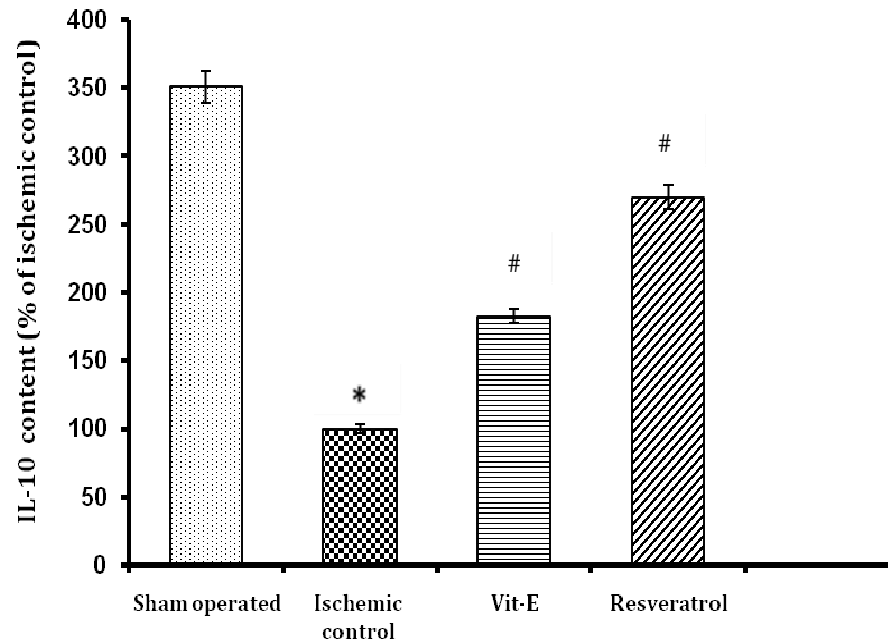


Figure 7. Effect of resveratrol and vitamin-E (Vit-E) on interleukin-10 (IL-10) content in the hippocampus tissue of rats subjected to Ischemia/Reperfusion (I/R) injury. All drugs were administered for 14 consecutive days, and on the 15th day ischemia followed by reperfusion were performed. Data was expressed as means \pm S.E. and as % of I/R control, $n = 8$. Statistical analysis was carried out by one-way ANOVA followed by The Tukey Kramer multiple comparisons test for comparison of means of different groups.

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failure, free radical production, excitotoxicity, altered calcium homeostasis, and activation of proteases (Choi, 1980; Panickar and Norenberg, 2005). Cerebral ischemia also turns down in the ability of brain mitochondria to function effectively thus affecting oxidative phosphorylation, a key mechanism of producing adenosine triphosphate (ATP).

Mitochondrial dysfunction may contribute to increased reactive oxygen species (ROS) production and lead to depolarization of the inner mitochondrial membrane potential. Such ischemia-associated changes can contribute to Ca^{2+} -induced membrane damage as well as increases in the Ca^{2+} -induced proteases, free radical mediated cell damage including membrane lipid peroxidation, and DNA damage (Sims and Muyderman, 2010). Minimizing oxidative stress and mitochondrial damage may result in reduced cell damage and a consequent improvement in cell viability following cerebral ischemia.

Increasing evidence supports the hypothesis that plant polyphenols provide protection against neurodegenerative changes associated with cerebral ischemia (Simonyi et al., 2005). Phenolic antioxidants have been shown to inhibit oxidative stress, a key event in the pathogenesis of cerebral ischemia, that damage

lipids, proteins, and nucleic acids, thereby inducing apoptosis or necrosis. Most studies have reported the protective effects of polyphenols in the hippocampal and cerebral cortex regions in ischemia (Dajas et al., 2003; Halliwell, 2008).

Levels of LDH activity could act as the marks of energy metabolism changes in the injured tissues and their quantity and quality could directly affect body's energy metabolism. When tissues and organs were injured, LDH activity abnormally increased (Rodrigo and Bosco, 2006; Doganay et al., 2006). In the present experimental results, resveratrol reduced LDH activity an effect that can be clarified by that tissue injury and cell death due to I/R is a free radical-mediated process in which oxidative stress and lipid peroxidation results in loss of cell viability and increased LDH activity. Resveratrol had a protective effect against LDH increasing and lipid peroxidation (Chander et al., 2005). It was also reported that a resveratrol analogue, astringinin, strongly prevented myocardial ischemia and infarction through increasing nitric oxide (NO) and decreasing LDH levels in the carotid blood (Luzi et al., 2004). Therefore, resveratrol may play an important role in protection against I/R through the observed improvement of LDH activity after tissue trauma.

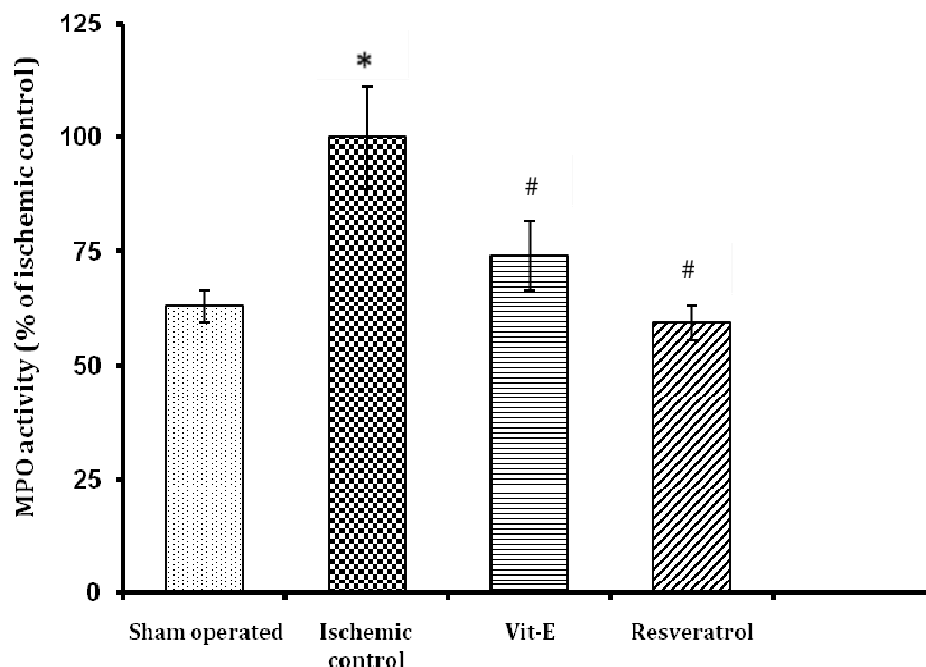


Figure 8. Effect of resveratrol and vitamin-E (Vit-E) on myeloperoxidase (MPO) activity in the hippocampus tissue of rats subjected to Ischemia/Reperfusion (I/R) injury. All drugs were administered for 14 consecutive days, and on the 15th day ischemia followed by reperfusion were performed. Data was expressed as means \pm S.E. and as % of I/R control, $n = 8$. Statistical analysis was carried out by one-way ANOVA followed by The Tukey Kramer multiple comparisons test for comparison of means of different groups.

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In the present work, the antioxidant potential of both vitamin E and resveratrol was shown through a decrease in lipid peroxidation measured as TBARS content. Vitamin E was found to reduce the volume of ischemic infarct (Garcia-Estrada et al., 2003; Mishima et al., 2003). In addition, administration of vitamin E alone or in combination with other vitamins increases the activities of antioxidant enzymes in rats following cerebral ischemia (Kashif et al., 2004). Gümüştaş et al. (2007) showed that administration of vitamin E before cerebral ischemia reduced lipid peroxidation and nitric oxide production in rats through free radicals scavenge properties of vitamin E (Gümüştaş et al., 2007).

Furthermore, resveratrol has been recently proposed as a potential antioxidant that could obviously inhibit free radical generation in several body tissues (Lu et al., 2013). In addition, the experiments *in vitro* also demonstrated that resveratrol could effectively protect DNA from oxidative damages, so as to assure cell proliferation, differentiation, and function to be normal (Jeong et al., 2014). Ray et al. (1999) found that resveratrol possessed cardio-protective effects through its peroxy radical scavenging activity and inhibiting lipid peroxidation (Ray et al., 1999).

Total antioxidant capacity was not altered in the resveratrol-treated group; this can be attributed to the fact that this parameter measures the combined enzymatic and non-enzymatic antioxidants capacity of the biological fluids. Thus, it provides an indication of the overall capability to counteract reactive oxygen species (ROS), resist oxidative damage and combat oxidative stress-related diseases. In some cases, the antioxidant effect may be mediated by counteracting the effect of certain enzyme which may potentiate the effect of another and thus the result is a non significant change.

I/R and associated oxidative stress up-regulates the expression of iNOS and increase NO_x concentrations (Huang et al., 2011). NO_x is synthesized during the stoichiometric conversion of L-arginine to L-citrulline in the presence of oxygen and nicotinamide adenine dinucleotide phosphate (NADPH), which is catalyzed by NOS (Moncada et al., 1991). Our results have indicated that I/R increased NO_x content in the hippocampus. This elevated NO_x content was not altered following resveratrol administration an effect that can be explained that shows that resveratrol up-regulates the expression of iNOS, indicating the ability of resveratrol to induce eNOS synthesis. In some tissue assays, resveratrol was found

to exert its protective action through up-regulation of NO synthesis thus it failed to neutralize the elevation of NO_x following I/R (Chander et al., 2005; Luzi et al., 2004).

In the present investigation, Global I/R was associated with inflammation indicated by elevation in both TNF- α and IL-6 hippocampal contents. Inflammatory activity is associated with increased levels of pro-inflammatory cytokines in the circulation, including IL-6 and TNF α (Bruunsgaard et al., 2001; Calabro et al., 2008). IL-6, along with TNF- α and IL-1 β , is known to play a key role in B-cell maturation and T-cell differentiation, as well as driving acute inflammatory responses (Schuett et al., 2012).

It has been observed that vitamin E suppresses inflammatory responses and oxidative damage induced by lipo-polysaccharide (LPS), a highly conserved cell wall component of Gram-negative bacteria known to initiate signaling cascade for inflammatory mediator expression including TNF- α , IL-6, and nuclear factor-kappaB, in both cell culture systems and animal experiments (Suntres and Shek, 1996; Takata et al., 1997; Berg et al., 2004). It was reported that α -tocopherol effectively prevented interferon-gamma/LPS-induced dopaminergic neuron degeneration (Shibata et al., 2006) and decreased LPS-induced lipid peroxidation and IL-6 in murine microglia and brain (Godbout et al., 2004).

Resveratrol reduced both TNF- α and IL-6 hippocampal content. Chen et al. (2013) demonstrated that resveratrol reduced joint swelling and cartilage destruction in adjuvant arthritis through inhibition of TNF- α production and showed that resveratrol down regulated the mRNA expression levels of the inflammatory factor, TNF- α (Chen et al., 2013). Furthermore, it was shown that treatment of diabetic rats with resveratrol significantly depressed TNF- α and IL-6 transcripts and the nuclear translocation of NF- κ B, suggesting an anti-inflammatory effect of resveratrol in the brain additionally. The anti-inflammatory effect of resveratrol has been studied in many other organs, including liver, heart, lung and kidneys (De la Lastra and Villegas, 2005; Docherty et al., 2005; Migliore and Coppede, 2009; Norata et al., 2007).

Conclusion

Briefly, this study demonstrates the beneficial influences of resveratrol on cerebral I/R through its antioxidant properties and inhibition of lipid peroxidation as well as through its anti inflammatory potentials.

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Conflicts of interest

The authors declared no conflicts of interest. This research received no specific grant from any funding agency in the public or commercial.

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