Original Communication

Neuroprotective Role of Trolox in Hippocampus after Ischemia Reperfusion Injury in Mouse

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Abstract: Cerebral ischemia is worldwide the third largest cause of mortality and disability in old people, and oxidative stress plays a considerable role in this process. In this study, for the first time, we evaluated the effects of Trolox as an antioxidative agent in ischemia induced by reperfusion. Twenty-four Syrian male mice were randomly divided into the 3 groups. Both common carotid arteries of Syrian mice were ligated bilaterally for 20 min, blood flow was restored and Trolox (50 mg/kg) was immediately injected after induced ischemia. Shuttle box results showed an improvement in memory in the Trolox group compared to the ischemia group, however, these improvements were not significant. Histopathological results showed a significant increase in the number of healthy cells in the hippocampal CA1 region in the Trolox group compared to the ischemia group ($p < 0.001$). Also, caspase-3, as an apoptosis marker, was significantly decreased in the Trolox group compared to the ischemia group ($p < 0.01$). Ultimately, as an anti-apoptotic factor, c-JUN was increased statistically in the Trolox group compared to the ischemia group ($p < 0.01$). Our study showed that after cerebral ischemia reperfusion, Trolox prescription increased anti-apoptotic proteins and decreased pro-apoptotic proteins thus protects neurons of the hippocampus and caused improvement of memory. Ultimately, these results would suggest some important treatment strategies after cerebral ischemia reperfusion.

Keywords: Trolox, hippocampus, ischemia, reperfusion

Introduction

Cerebral ischemia is worldwide the third largest cause of mortality and disability in old people. There is no satisfactory treatment for this issue [1]. The hippocampus surely is the part of the brain most sensitive to ischemia and hypoxia.

Hypoxia causes a reduction in ATP inhibiting synaptic potentials and is a mechanism to reduce energy consumption in hypoxic cells, which leads to cell death. The hippocampus plays an important role in transferring information from short-term memory to permanent memory. It has a key role memory consolidation, organizing and storing information associated with feelings and memories [2, 3]. Hence it is involved in memory formation, information processing and is among the first areas of the brain affected by degenerative diseases like Alzheimer, Huntington, Parkinson and by injuries from trauma or ischemia. Damage to the hippocampus leads to amnesia and loss of memory later in life, earlier memories, however are maintained and can be remembered [4]. Bilateral damage to the hippocampus can cause subsequent or progressive amnesia [5].

Oxidative stress is considered to be involved in a number of human diseases, including ischemia. Primary studies confirmed the reduction of brain cell death following treatment with antioxidants [6]. Although vitamin E has many biological functions, its antioxidant role is the most significant part. As shown before, vitamin E supplementation can alleviate lipid peroxidation in aged diabetic rat brains [7]. It also relieves lipid peroxidation in the brain which could initiate some processes that participate in the

progression of Alzheimer's disease [8]. Another function of vitamin E might be in cell signaling [9]. Tocopherol has shown to enhance the numbers and survival rates of granule cells in the dentate gyrus of rats [10]. Trolox is a water-soluble form of vitamin E. It is an antioxidant like vitamin E, used in biological or biochemical applications to reduce oxidative stress or damage [11].

Based on the information provided, we have investigated the neuroprotective role of Trolox in ischemia reperfusion damages.

Materials and methods

Animals

24 Syrian male mice weighing 25–35 grams provided by the Iranian Razzi Institute were kept in a controlled environment (humidity and temperature) on a 12/12-h light/dark cycle with free access to standard food and water. The animals were randomly divided into 3 groups (8 animals in each group). All efforts were made to minimize the number of animals used and making them less agonized. Sham group: received a surgery stress (without ischemia reperfusion); control group: received an ischemia reperfusion injury after surgery process; Trolox group: received Trolox by intra-peritoneal injection at a dose of 50 mg/kg after performing surgery and ischemia reperfusion injury.

Ischemia reperfusion injury modeling

All animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Skin incision was performed on the midline of the ventral neck. Both common carotid arteries were ligated bilaterally using micro-surgical clips for 20 min, subsequently clips were removed and blood flow was restored. The skin incision was sutured and the animals were taken care of in an adequate condition until recovery, their body temperature kept at 37.5 °C with a heating pad.

Behavioral test (shuttle box)

Ten days after ischemia/reperfusion, the animals were evaluated by shuttle box. The box was divided into bright and dark sections with defined dimensions (20 × 30 × 20 cm) and compartmented by a guillotine door placed in the illuminated compartment. Then, after an adaptation period in the bright compartment (30 s), the guillotine door was opened to allow the animal to enter the dark compartment. When the animal entered the dark compartment, the guillotine door was closed and an electrical foot shock (0.25 MAh, 20 V) was delivered through the grid floor for 2 s. The time it took to enter the dark compartment (latency) was recorded and after 24 h the trained animals were placed in the bright compartment and the latency time of their entry into the dark compartment was recorded for up to 300 s.

Histopathological assay

Nissle staining was used for the detection of Nissle bodies in the cytoplasm of neurons. This method is commonly used to identify the basic structure of necrotic neurons in the brain and spinal cord. The brains of 4 animals of each group were perfused with 4% paraformaldehyde, removed from the skull and fixed by immersion in the same fixative for at least 2 days before histological processing. The parietal cortex was dehydrated and embedded in paraffin. A series of layers with a thickness of 10 μM was cut (coronally) and stained with crystal violate. Nissle bodies were colored purple-blue.

Western blotting

After sacrificing the animals under anesthesia, brains were sampled and hippocampi placed into lysis buffer on ice (RIPA buffer: Sigma R027, Protease Inhibitor Cocktail: Sigma P8340). Tissue samples were homogenized at 40 °C and placed on the ice for 45 min, then centrifuged for 20 min at 1200 RPM at 4 °C. Supernatants were collected and assayed for total protein (Radford method). After extraction of total protein it was mixed with denaturizing loading buffer (basic Tris pH 6.8, 1/25 mL, glycerol 1 mL, SDS 0.2 g, mercaptoethanol 0.1 mL), loaded on a G10 SDS-polyacrylamide gel and ran at 75V for 15 min before running at 150 V for 60 min at RT. The gel was transferred onto the nitrocellulose membrane at 135 MAh for 1.5 h at 4 °C. The membrane was blocked 1 h later with 3% non-fat dry milk in TBS and then incubated with the mouse antibody of caspase-3 and c-JUN (G10 Abcam) diluted 1:1000 for 2 h at RT. The immune-reactive band was detected by using a peroxidase-conjugated goat anti-mouse immunoglobulin G diluted 1:5000 for 1 h; followed by the application of the ECL plus chemiluminescence western blotting kit (Amersham, ECL).

Statistical analysis

The statistical comparisons between groups were carried out using ANOVA, followed by the Tukey's test for post hoc analysis. Statistical analysis was performed using SPSS

version 15. P < 0.05 was accepted to denote statistically significance and all data were presented as mean ± SEM.

Results

Behavioral test

The behavioral test gave the Step-Through Latency (STL) time in the Sham group with 19.71 s before the shock and 298.86 s after the shock. Ischemia group: 18.17 s before and 137.86 s after the shock. Trolox group: 22.33 s before and 254 s after the shock. These results showed that the STL time after the shock was decreased significantly in the ischemia group compared to the sham group (p < 0.05). In the Trolox group it did not increase significantly compared to the ischemia group (Figure 1, 2).

Histopathological assay

The average number of euchromatin cells in the CA1 region of the hippocampus in the ischemic group (43.53) was significantly decreased compared to the Sham group (89.47; p < 0.01). The cell density in the Trolox group (65.07) was significantly higher than in the ischemic group (p < 0.001) (Figure 3, 4).

Western blotting

Some proteins increase during inflammation and in necrotic conditions. Western blotting showed that the amount of caspase-3 protein in the ischemic group (40090.67 pixel) increased significantly compared to the sham group (25485 pixel; p < 0.001). In the Trolox group (32457.33 pixel) caspase-3 decreased significantly compared to the ischemia group (p < 0.01; Figure 5A). The amount of c-Jun protein in the ischemic group (65.69 pixel) increa-

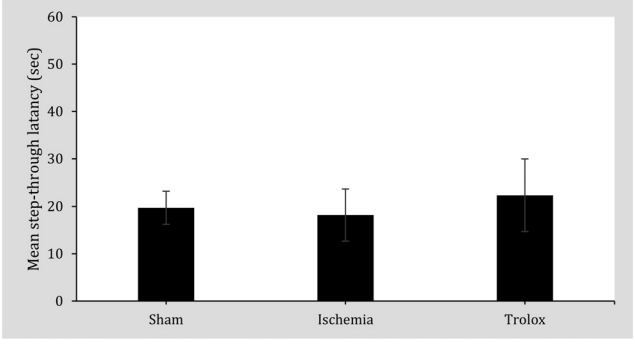

Figure 1. Step Through Latency (STL) time before the shock. There are no diffrences between the groups. Data are shown as Mean ± SEM.

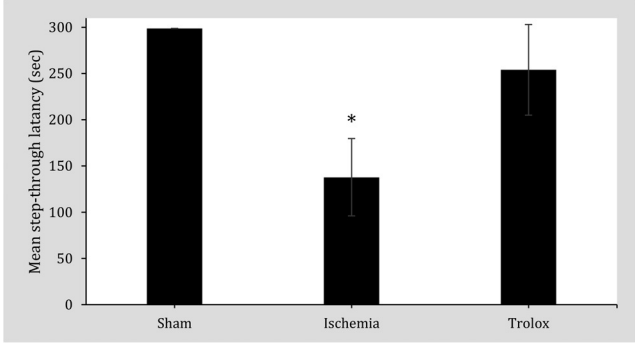

Figure 2. Step Through Latency (STL) time after the shock. There is a significant diffrence between Sham and Ischemia group. STL decreased in the ischemia group but in Trolox group it increased significantly. Data are shown as Mean ± SEM. * p < 0.05.

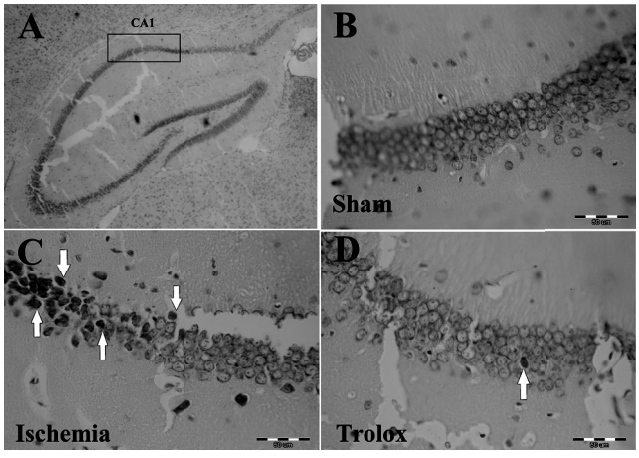

Figure 3. Nissle staining result in 3 groups. In this staining method the necrotic cells are indicated with dark and compact nucleus (Arrowhead). **(A)** The Sham group (low magnification). **(B)** The Sham group (high magnification) without necrotic cells. **(C)** The ischemic group with a lot of necrotic cells (Arrowhead). **(D)** The Trolox group with infrequent necrotic cell than Ischemic and Sham group.

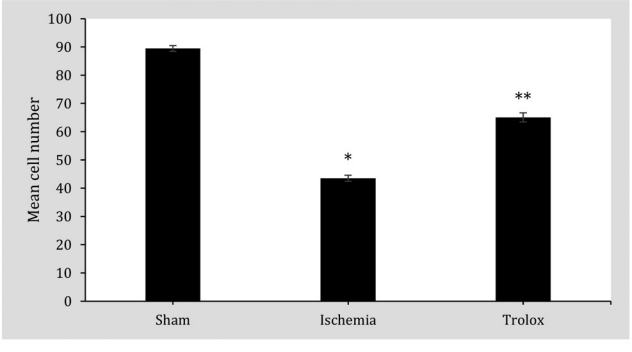

Figure 4. Comparison of the density of healthy cells in the CA1 region of the hippocampus in 3 groups. The cell density of the Sham group is significantly different to Ischemic group. The cell density of the Trolox group is significantly different to Ischemic groups. Data are shown as Mean ± SEM. * p < 0.001. ** p < 0.01.

sed significantly compared to the sham group (5258 pixel; $p < 0.01$) and along with that, it increased significantly in the Trolox group (8084 pixel) compared to the ischemia group ($p < 0.01$; Figure 5B).

Discussion

Ischemia reperfusion injury is known to damage the brain via oxidative stress. Modeling it by ligating both common carotid arteries was used in other experiments to study the brain and its damage or improvement, and discussed in neuroprotection and other fields [6]. A water-soluble form of vitamin E, Trolox, is used in biological or biochemical applications to reduce oxidative stress or damage that takes place in some tissues and organs from skin to central nervous system. Vitamin E can improve skin diseases such as dermatitis [12] and is an anti-ageing agent. Kabay et al. revealed neuroprotective effects of vitamin E on the ischemic damage in diabetic central neuronal cells [13]. They assayed histopathological changes with hematoxylin and eosin techniques. Kana et al. observed that vitamin E decreases genotoxicity and the histopathological changes induced by deltamethrin [14].

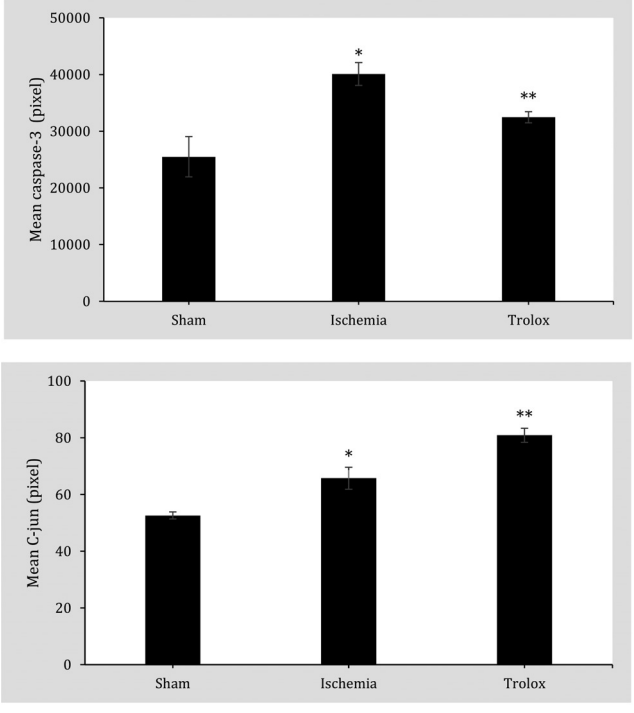

Figure 5. Western blotting of caspase-3 and c-Jun protein. Caspase-3 (*p < 0.001**)** and c-Jun (*p < 0.01) protein in the Ischemic group was increased compared with the sham group significantly **(A** and **B,** respectively**). A)** Caspase-3 protein in the Trolox group was decreased compared to the ischemia group significantly (**p < 0.01)**. B)** C-Jun protein in the Trolox group was increased compared to the Ischemia group significantly (**p < 0.01)**.** β-actin expression in all groups **(C)**. Data are shown as Mean ± SEM.

Trolox, memory and learning

Our findings confirmed an improvement of memory in the Trolox group; however, this improvement was not significant (Figure 1, 2).

The effects of vitamin E and its homologues on memory and learning have been widely investigated. Administration of vitamin E in aging rats has dramatically improved the function of memory [15]. When vitamin E is administered for Alzheimer's disease, the symptoms of dementia are significantly delayed [16].

Co-administration of vitamins E and C in diabetic rats has shown to improve memory and learning [17]. Co-administration of vitamin E and melatonin significantly improved learning and memory in diabetic rats [18]. Trolox is a potent antioxidant that prevents the propagation of free radicals, thereby preventing oxidative stress. Recent studies have shown that vitamin E prevents suppression of antioxidant enzyme systems such as glutathione peroxidase (GPX), catalase and superoxide dismutase (SOD) [19]. Thus, vitamin E may improve memory and learning impairment due to its natural antioxidant property.

Possible mechanisms by which oxidative stress causes damage to learning and memory are not fully understood. In this matter, it could be due to signaling molecules such as cAMP response element-binding protein (CREB) and $Ca2^{+}$/calmodulin-dependent protein kinase IV (CAMKIV), which are affected by oxidative stress. A recent study has shown that oxidative stress is associated with decreased levels of these two phosphorylated molecules [20]. Both molecules are essential for the proper function of memory [21, 22]. Thus, it is possible that increased oxidative stress during ischemia reperfusion injury leads to the suppression of crucial signaling molecules such as CREB and CAMKIV. These molecules play a critical role in memory function. Trolox is a potent antioxidant that can inhibit this cascade of events; therefore it could prevent damage to learning and memory.

Trolox and cell density

During ischemia reperfusion, free radicals cause tissue damage and cell death [23–27]. Hippocampal CA1 pyramidal cells are the most sensitive cells in the brain during ischemia and reperfusion injury [28, 29]. After ischemia followed by reperfusion, oxygen radicals are the main cause of damage to hippocampal CA1 cells [30]. Free radicals cause

cell membrane lipid peroxidation. Gupta et al. have shown that Trolox has a neuroprotective role and also reduces cell death in the CA1 region of the hippocampus. According to their study, Trolox protects the neurons of the hippocampus by reducing lipid peroxidation [31]. Results of Wang et al. and Park et al. have shown that a decrease in free radicals with Trolox treatment is responsible for a reduction of neuronal death [32, 33]. In our study also, Trolox significantly increased the number of healthy cells in the CA1 area compared to the ischemic group. Although oxidative stress and lipid peroxidation status were not assessed in our study, according to Wang et al. and Park et al., an increment of the number of healthy CA1 cells in our study could be related to the inhibition of free radicals and reduction of lipid peroxidation. Trolox collects free radicals and also protects intracellular structures such as mitochondria and lysosomes [30, 34]. Studies have clearly shown that the administration of vitamin E decreased hemorrhage, necrosis, neuronal damage, and infiltration of macrophages in the parenchyma and in perivascular spaces [35]. Vitamin E reacts with free radicals and dramatically prevents apoptosis in hippocampal cells [30].

Trolox and caspase-3

This study showed that the expression of caspase-3 in the treatment group decreased significantly compared to the ischemia group (Figure 5). These results are concordant with results of similar studies. Wu et al. showed that vitamin E inhibits the activity of caspase-3 and thereby inhibits apoptosis. Their results showed that intracellular antioxidants such as vitamin E are able to tolerate the intracellular redox status, reduces caspase-3 activity and inhibits apoptosis-related mitochondrial signal transduction [36]. In another study, Wu et al. showed that N-acetyl-cysteine and vitamin E suppress the production of reactive oxygen species (ROS) by preventing caspase-3 activity and also prevent the release of cytochrome C and SMAC/DIABLO from mitochondria into the cytosol, ultimately preventing apoptosis [37]. Reduced expression of caspase-3 and subsequently decreased apoptosis could be the explanation for the improved memory and cell density in the Trolox group.

Trolox and c-Jun

C-Jun and its dimerization partners are subject to regulation by an incredibly diverse array of extracellular stimuli such as peptide growth factors, pro-inflammatory cytokines, oxidative and other forms of cellular stress (ischemia reperfusion) [38]. In this experiment, the c-Jun protein level in the Trolox group was increased significantly compared to the ischemia group, which is compatible with previous studies. The levels of c-Jun mRNA and protein in vitamin E-treated cells were also increased. In another study, the expression of c-Jun was prolonged by vitamin E [39]. Phenolic antioxidants specifically induce expression of c-Jun. After treatment of quiescent human hepatoma HepG2 cells with phenolic antioxidants, such as vitamin E, the level of c-Jun mRNAs is substantially increased [40]. C-Jun is required for proliferation. Wang et al. showed that cyclin D1 and D3 are the main targets of c-Jun in controlling the process of cell proliferation [41]. C-Jun is an anti-apoptotic protein. The molecular mechanisms by which c-Jun protects cells from apoptosis are unclear. One possibility is that c-Jun might participate in a checkpoint function and mediates a growth arrest function that permits repairing damaged DNA [38]. A second possibility is that c-Jun induces the expression of genes that block apoptosis. This would be functionally analogous to the mechanism by which NF-κB protects cells from TNFα-induced apoptosis, which involves transcriptional activation of the anti-apoptotic gene cIAP2 [42]. The anti-apoptotic role of c-Jun and its impact on the proliferation process could be a reasonable explanation for the c-Jun increase in brain tissue after ischemia reperfusion. This may be a compensatory mechanism to protect brain tissue after ischemia reperfusion. Our study showed that the level of c-Jun protein in the ischemic group was increased significantly compared to the sham group. Finally, in our study, improved memory and increased cell density in the Trolox group, according to the explanations above, are consistent with the increase in c-Jun protein due to its anti-apoptotic function.

Conclusion

This study concludes that after cerebral ischemia reperfusion, Trolox prescription protects the neurons of the hippocampus by increasing levels of anti-apoptotic proteins and decreasing levels of pro-apoptotic proteins, so it may improve memory. These findings would be important for choosing a proper treatment strategy after cerebral ischemia reperfusion.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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