Evaluation of the Effect of Resveratrol on Hippocampus in Experimental Traumatic Brain Injury

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Abstract

Aim: Many antioxidant compounds have been tried to prevent traumatic brain injury (TBI). In this study, we aimed to demonstrate the therapeutic effect of Resveratrol in the hippocampus in TBI by histopathologic and immunologic evaluation.

Study Design: Twenty-four male Sprague-Dawley rats were divided into Control, TBI, TBI+Resveratrol (20 mg/kg/day, oral) groups. Rats were subjected to traumatic brain injury by dropping the weight from a height with a 50 g/1m weight-height impact device. All rats were decapitated 14 days after trauma induction and the protective effects of Resveratrol were evaluated by histopathological and immunohistochchemical analyses.

Result: In the TBI group, degeneration of cells, dilatation of vessels and apoptosis due to traumatic inflammation were observed in the alveus and pyramidal layer. In the plexiform layer, synaptic degeneration was present in nerve extensions. In TBI+Resveratrol group, vascular dilatation was decreased and axonal extensions were normal, hyperplasia was observed in pyramidal neurons. S100 expression was positive in pyramidal neurons, glial cells and vascular endothelium. In the Resveratrol treated group, negative expression was observed in membranes, pyramidal neurons and positive s100 expression was observed in plexiform layer and axons.

Conclusion: We suggest that after TBI, Resveratrol treatment alleviates cerebral tissue pathology and can be demonstrated by s100 expression in neuronal regeneration.

Keywords: Traumatic brain injury, hippocampus, s100, Resveratrol, immunohistochemistry

INTRODUCTION

Traumatic brain injury (TBI) is one of the leading causes of morbidity, disability and mortality at all ages 1. As of 2005, approximately 3.17 million TBI survivors experience post-traumatic complications ranging from neurologic and psychosocial problems to long-term chronic conditions 2. Traumatic brain injury causes primary and secondary changes in neuronal tissues. Primary damage occurs as a result of mechanical damage to neuronal extensions such as blood vessels, nerve cells, axons and dendrites that occur at the moment of sudden impact. Secondary damage begins a few minutes after the primary damage and may last for months as a result of a series of metabolic, cellular and molecular events leading to neural tissue damage and brain cell death. This process may be accompanied by an increase in free oxygen radicals, an increase in neurotoxic glutamate, and an increase in neuronal calcium and sodium 3. Increased understanding of the clinical picture of TBI and the underlying complex pathophysiologic mechanisms has led to the development of new and promising therapeutic approaches that have shown promising effects in preclinical studies and phase I/II trials 4,5.

The hippocampus is a layer of gray matter, located in the medial part of the temporal lobe and is in close neighborhood with the temporal horn of the lateral ventricle. Neural plasticity in the hippocampus continues throughout life 6. Its function has been investigated for many years, but both the complexity of its structure and its close relationship and intensive connection with many regions in the brain make it difficult to fully explain its function. It is known that the hippocampus is related to memory, especially short-term memory 7.

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) plays an important role in scavenging free radicals 8. In vivo, Resveratrol shows its antioxidant properties more as a gene regulator. Resveratrol inhibits NADPH oxidase-mediated ROS production by down-regulating the expression and activity of the oxidase enzyme. This polyphenolic compound reduces mitochondrial superoxide formation by stimulating mitochondrial biogenesis. Resveratrol prevents superoxide production from unbound endothelial nitric oxide synthase by up-regulating GTP cyclohydrolase I, the enzyme that synthesizes tetrahydrobiopterin. In addition, Resveratrol increases the expression of several antioxidant enzymes. Some of the gene regulatory effects of Resveratrol are mediated through histone/protein deacetylase sirtuin 1 or nuclear factor-E2-related factor-2 9-10. The S100 protein family constitutes the largest subgroup of the calcium-binding protein group. S100 proteins are acidic...
proteins of 10-12 kDa. The S100B protein subgroup is neurotrophic at physiologic concentrations and neurotoxic at high concentrations, and its immunohistochemical expression or serum levels have been detected in various pathologic conditions. S100B has also been reported as a marker of astrocytic activation. Members of the S100 protein family are multifunctional proteins expressed in a wide variety of tissues. They are involved in the regulation of various cellular processes such as contraction, motility, cell growth and differentiation, cell cycle progression, transcription, structural organization of membranes, dynamics of cytoskeletal components, protection from oxidative cell damage, protein phosphorylation and secretion through interactions with various effector proteins in cells.

In this study, considering the protective effects of resveratrol shown in many studies, we aimed to investigate the efficacy of Resveratrol against the damage in the hippocampus region of the brain in traumatic brain injury.

MATERIALS AND METHODS

Resveratrol (Sigma Chemical Company, St. Louis, MO, USA) and s100 antibody (Santa Cruz Biotechnology Inc. CA, USA) were used in the experiment. Before starting the experimental procedure, the experimental animals were anesthetized intramuscularly with 90 mg/kg ketamine hydrochloride (Ketalar; Pfizer, Istanbul, Turkey) and 8 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey). Three groups were formed with eight rats (n=8) in each group and the following procedures were applied to the groups.

1. Control group: After anesthesia, all rats were fixed on the operation table in the prone position, the temporal region of the calvarium was shaved and cleaned with 10% povidone-iodine and opened with a 3 cm lower midline incision. The surrounding connective tissue was shifted backwards to expose the temporal bone. The scalp was sutured without any other procedure. For 14 days, the animal was placed in a normal cage and observed.

2. TBI group: After anesthesia, all rats were fixed on the operation table and in the prone position, the temporal region of the calvarium was shaved and cleaned with 10% povidone-iodine and opened with a 3 cm lower midline incision. The surrounding connective tissue was shifted back to expose the temporal bone. A steel cap was placed over the temporal bone of the rat. Then, a 50 g weight made of brass with a diameter of 18 mm was dropped from a plexiglass tube with a height of 1 meter, causing trauma to the temporal bone. A steel cap was placed over the temporal bone. Then, a 50 g weight made of brass with a diameter of 18 mm was dropped from a plexiglass tube fixed on a ring stand from a height of 1 meter, causing trauma to the skull. The scalp was sutured and 20 mg/kg/day Resveratrol was given orally for 14 days.

On the 14th day after the end of the experimental phase, the animals were sacrificed by intracardiac blood sampling under general anesthesia. The calvaria of the rats were removed and the brain tissue was excised and kept in zinc-formalin solution. After fixation in zinc-formalin for 72 hours, routine paraffin tissue processing was performed. From the paraffin blocks obtained, 5 micron sections were taken with a rotary microtome and stained with Hematoxylin-Eosin to examine tissue histopathology.

Immunohistochemistry

5 µm thick sections taken from paraffin blocks on slides were kept in an oven at 58-62°C for 6 hours to melt the excess paraffin. Then, the sections were cleared of paraffin by soaking in xylene for 3x15 minutes. The sections were rinsed in a decreasing series of alcohol (100%, 96%, 90%, 70%, 50% ethyl alcohol) for 10 minutes each and soaked in distilled water for 5 minutes. Sections were washed in phosphate buffer solution (PBS) for 3x5 minutes. The sections were placed in EDTA buffer solution (pH: 8.0, catalog no: ab93680, Abcam, Cambridge, USA) and heated-induced epitope retrieval was performed. Then, the sections were left at room temperature for 20 minutes and taken back into PBS. Hydrogen peroxide solution (catalog no: TA-015-HP, ThermoFisher, Fremont, CA, USA) was added to the sections and repeated after 10 minutes for a total of 20 minutes. Then washed again with PBS for 3X5 minutes and kept in Ultra V Block (catalog no: TA-015-UB, ThermoFisher, Fremont, CA, USA) solution for 7 minutes. Sections were overnight with S100 antibody at +4°C overnight. The next day, the sections were kept at room temperature for 1 hour, washed with PBS and incubated with biotinylated secondary antibody (catalog no: TP-015-BN, ThermoFisher, Fremont, CA, USA) for 14 minutes. Then streptavidin-peroxidase (catalog no: TS-015-HR, ThermoFisher, Fremont, CA, USA) was added and incubated for 15 minutes. Diaminobenzidine (DAB) (catalog no: TA-001-HCX, ThermoFisher, Fremont, CA, USA) was added on the sections. The tissues were kept in PBS for 15 minutes and then counterstained with Harris hematoxylin. The sections were then covered with entellan (catalog no: 107961, Sigma-Aldrich, St. Louis, MO, United States) and imaged by Zeiss Imager A2 photomicroscope.

RESULTS

Histopathologic findings

In the section of the control group, it was observed that the extensions of the pyramidal cells were regular and extended towards the plexiform layer. Slight dilatation was observed in the vessels. The nuclei of some cells were reduced in size and axon hillocks were regular. Axons in the plexiform layer were parallel to each other (Figure 1a). In the sections of the TBI group, the cytoplasm in the alveus and pyramidale layers of the hippocampus was empty and vacuolar. Variability in the vessels. The nuclei of some cells were reduced in size and axon hillocks were observed prominently. Axon hillocks were observed prominently. In the plexiform layer, it was observed that the extensions were parallel to each other and dilatation of the vessels decreased (Figure 1c).

Immunohistochemical findings

In the immunohistochemical examination of the control group, a chromatin-rich nucleus structure was observed especially in the alveolar and pyramidal layers of the hippocampus. The membrane integrity of granulated cells was preserved especially in the alveolar and pyramidal layers of the hippocampus. Their nuclei were small. Vessels were observed to be regular.
S100 positivity was found to be moderate especially in the plexiform layer. Negative S100 expression was observed in pyramidal cells and granulated cells (Figure 1d). In the immunohistochemical examination of the TBI group, positive S100 expression was detected in pyramidal neurons with degenerative changes. Some glial cells showed positive S100 expression. In general, it was observed that the layer integrity started to deteriorate and detach. S100 expression was found to be positive in the endothelium of blood vessels due to trauma effect. S100 expression was especially strong in synaptic regions, plexus parts and plexiform layer (Figure 1e). In the immunohistochemical examination of TBI + Resveratrol group, S100 expression was negative in membranes, especially in primidal neurons and granulosa cells. Positive S100 expression in glia cells continued in this group. The degenerative features of primidal neurons were observed to persist. Positive S100 expression was observed in axons. It can be said that S100 positivity continued in the plexiform layer but S100 proteins were negative at the cellular level (Figure 1f).

**Figure 1.** (a) Control group: vessels are slightly dilated and regular (black arrow), axon hillock areas are regular (arrowhead), axonal structures (asterisks) in the plexiform layer are parallel and upward (H-E staining); (b) TBI group: cytoplasm of nerve cells empty and vacuolar (black arrow), degeneration and loss of nucleus in pyramidal cells (arrowhead), dilatation of vessels (green arrow), localized edema in glomus-like areas (asterisk), reduction in synaptic structures (red arrow) (H-E staining). (c) TBI + Resveratrol group: smooth membrane (black arrow), pyknosis in nuclei (arrowhead), regular axonal extensions (red arrow), mild hyperplasia in pyramidal neurons (green arrow) (H-E staining). (d) Control group: positive S100 expression in plexiform layer (asterisk), negative S100 expression in pyramidal cells and granular cells (black arrow) (S100 immunostaining). (e) TBI group: Positive S100 expression in pyramidal neurons (black arrow), glial cells (asterisk), endothelial layer of blood vessels (yellow arrow), plexiform layer (orange star) (S100 immunostaining). (f) Trauma+Resveratrol group: negative S100 expression in pyramidal neurons (black arrow), positive S100 expression in glia cells (yellow arrow) and plexiform layer (asterisk) (S100 immunostaining).

**DISCUSSION**

Traumatic brain injury (TBI) is a major cause of mortality and disability worldwide. It may start and continue with mild symptoms or may cause severe neuropathologic damage and neurologic dysfunction. The blood brain barrier is damaged by TBI and many cellular dysfunctions such as inflammation, edema and disruption of the vessel wall occur in the brain. TBI causes an increase in reactive oxygen radicals, peroxidation of cellular and vascular structures and depletion of the endogenous antioxidant system. As a result of oxidative stress after TBI, many activated cellular and molecular pathways are involved in damage in vascular endothelial cells, astrocytes and microglia, and various neurons.

Many plant ingredients rich in polyphenols have shown promising effects in TBI. Resveratrol is one of the natural polyphenolic compounds derived from many fruits such as grapes and strawberries. Previous studies have shown a wide range of bioactivities of Resveratrol, including anti-oxidative, anti-apoptotic, anti-inflammatory and neuroprotective activities. For example, Resveratrol has been proven to reduce neuronal apoptosis and decrease the levels of inflammatory factors such as interleukin (IL)1β, IL10 and tumor necrosis factor (TNF)α in spinal cord injury. Resveratrol has previously been shown to protect against various pathological conditions, including the brain in brain trauma. In an experimental rabbit brain injury study by Singleton et al. Resveratrol was found to protect cortical contusion and hippocampal damaged neurons.

In our study, cell cytoplasm was vacuolated in the alveolar and pyramidal layers of the hippocampus in the TBI group. Degeneration, nucleus loss and dilatation of vessels were observed in pyramidal neurons. There were synaptic degenerations in the form of gaps in the nerve extensions in the plexiform layer and pyknosis in the nuclei of pyramidal neurons. In TBI+Resveratrol group, membrane integrity was preserved, pyknosis in nuclei continued in some areas, and vessels and axonal extensions were regular. While hyperplasia was observed in some pyramidal neurons, plexiform layer extensions were parallel to each other (Figure 1b,c).

S100 proteins are a family of low molecular weight calcium binding modulator proteins. In the brain, S100 protein is predominantly produced by astrocytes and its enhanced synthesis indicates activation of astrocytes in response to damage to neural tissue. Extracellular S100B can exert neurocytotoxicity for neurons and glia cells by inducing both apoptosis and cell necrosis. The toxic effect is based on the ability of S100 to induce proinflammatory cytokines, oxidative stress enzymes, especially iNOS. The development of destruction in TBI is largely associated with inflammatory processes in the brain. Overexpression of S100B in neuronal structures is responsible for the initiation of the gliotic reaction by releasing proinflammatory molecules that may have a detrimental effect on other cells. Genrikhs et al. showed that astroglial S100 protein expression decreased in Methylene blue treatment after TBI.

In our study, in the immunohistochemical examination of the TBI group, positive S100 expression was found in glial cells and blood vessel endothelium with degeneration in pyramidal neurons. S100 expression was especially severe in synaptic regions and plexiform layer. In TBI+ Resveratrol group, S100 expression was negative in pyramidal neurons and granulosa cells. Degenerative changes in pyramidal neurons and positive expression in glial cells and axons continued in this group. It can be said that S100 positivity persisted in the plexiform layer but S100 proteins were negative at the cellular level (Figure 1e,f).

**CONCLUSION**

Resveratrol has many biological activities. In this study, it was observed that the neuroprotective effect of Resveratrol was effective after TBI, and it showed this by decreasing s100 beta expression. With this study, we believe that neurological pathologies that may occur after TBI injury can be histologically improved with Resveratrol treatment.
CONFLICT OF INTEREST
The author declared that there was no conflict of interest during the cause of this study and producing and submitting this manuscript for publication.

Author contribution
E.D., F.A., I.S.E., S.O.B., O.K., M.N.F. and A.A. contributed equally to manuscript drafting, writing, data collection, conceptualization and observation. All authors read and approved the final version of the manuscript.

Data availability
All generated data were presented in this study.

Ethical approval
Ethical approval was taken from Dicle University, Animal Experiments Local Ethical Committee (date: 07/04/2021 and number: 2021/18).

Funding
This study was funded by Dicle University Project Research Platform (DUBAP) with project number: TIP.22.018

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