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Fırat Aşir et al., Resveratrol and hippocampus relationship

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ABSTRACT

Background: Traumatic brain injury (TBI) is the damage to the brain caused by external blow or jolt to the head or body. TBI secondarily induces cell damage in the hippocampus. This study aimed to investigate effects of resveratrol treatment histological examination and nuclear factor kappa B (NFκB) expression in hippocampus after TBI.

Materials and methods: Twenty-four rats were assigned to three groups: sham, TBI and TBI+Resveratol. TBI was conducted by dropping a 50-g weight from a 1-meter height from a tube to the head of animals. 20 mg/kg resveratrol was orally administered to rats after TBI. Blood was collected to measure malondialdehyde (MDA) and glutathione (GSH) contents. Cerebral tissues were processed for histopathology and furtherly for immunohistochemical analysis.

Results: MDA content was significantly increased and GSH value were significantly decreased in TBI group compared to sham group. Resveratrol treatment significantly improved biochemical scores in TBI+Resveratrol group. Normal histological appearance was observed in hippocampal sections of sham group. In TBI group, neurons in hippocampus were

degenerated. Their nuclei were pyknotic. Other neurons and supportive neuroglial cells in hippocampal proper and dentate gyrus were also disrupted. Hippocampal proper integrity was lost with vascular dilatation. NFκB was upregulated in hippocampal neurons of TBI group. **Conclusions:** Resveratrol treatment alleviated pathologies and downregulated NFκB expression in hippocampus. TBI caused adverse alterations in free radicals' balance system and histological structures of hippocampus. Resveratrol with its antioxidant and anti-inflammatory effects reduced the damage caused by TBI.
Key words: trauma, hippocampus, inflammation, neurons, rat

INTRODUCTION

Traumatic brain injury is defined as a dysfunction in the structure and functions of the brain tissue as a result of a strong external blow to the head or body [1,2]. TBI can be permanent or temporary. TBI is an important cause of morbidity and mortality in individuals of all ages. Globally, annual incidence of TBI is 790 per 100,000, constituting more than 50 million TBI cases [1-3]. TBI can be primary and secondary brain injury. In primary brain injury, the neural tissues and cells of the brain are directly damaged. At this stage, cell death, vascular disorders, and disruption of tissue integrity occur. In secondary brain injury, events develop following the primary brain injury. The treatment of TBI varies according to the cause, severity, and anatomic location of the injury. Physical rehabilitation therapy or medication therapy is recommended in mild cases [4-7].

Medicinal plants have been used in traditional medicine for centuries in the treatment of various diseases. Historically, different organs or extracts of plants have been tested on numerous diseases. There are various studies on the efficacy of medicinal plants [8-10]. Resveratrol is a natural stilbenoid found in dozens of plant species such as grape skin, blueberry, raspberry, mulberry, and peanut [11]. Resveratrol is biologically active compound with antioxidant properties due to its polyphenol ring [12,13]. Moreover, it has anti-inflammatory, immunomodulatory, anti-aging, anti-cancer, cardioprotective, neuroprotective, antidiabetic properties [14-18]. Resveratrol communicates with many enzymes and receptors inside the cell. Resveratrol especially interferes with the inflammation pathway.

Nuclear Factor kappa B (NFκB) is a key transcription factor and expressed in almost all cell types. NFκB is a proinflammatory molecule and involved in the activation of inflammatory genes, cytokine and free radical production, viral/bacterial infections, and cell survival. NFκB has an important role in TBI due to induction of inflammatory events.

Present study aimed to investigate the efficacy of resveratrol on hippocampus with oxidative stress and histochemical perspective after TBI.

MATERIAL AND METHODS

Ethical approval and animal housing

All animal experiments were approved by local permission of Dicle University Animal Experimentation Local Ethics Committee (2021/18). Animals were allowed to access to water and food ad libitum and housed in cages (12/12 dark/light period, 23±1°C). Resveratrol was purchased from Merck (catalog no: R5010, Germany).

Surgical procedures

All procedures were performed under anesthesia. Twenty-four Wistar albino female rats (12 weeks old, weighing 200-250 g) were assigned to three groups: sham, TBI, TBI+Resveratrol (n:8 per group).

Sham group: All rats was fixed on the operating table after anesthesia as prone position. The temporal region of the calvarium was shaved and cleaned with 10% povidone-iodine. 3 cm lower midline incision was opened, and the surrounding connective tissue was removed. The scalp was sutured without any further intervention. The animals were placed back to its normal cage for 24 hours.

TBI group: All rats were fixed on the operating table after anesthesia as prone position. The temporal region of the calvarium was shaved and cleaned with 10% povidone-iodine. 3 cm lower midline incision was opened, and the surrounding connective tissue was removed. Trauma was conducted by dropping a 50 g weight made of 18 mm diameter brass from a height of 1 meter from a plexiglass tube fixed to the stand. The scalp was sutured and 0.1 ml 0.9% saline i.p. only once. The animal was placed in its normal cage and observed for 24 hours.

TBI+Resveratrol group: All rats were fixed on the operating table after anesthesia as prone position. The temporal region of the calvarium was shaved and cleaned with 10% povidone-iodine. 3 cm lower midline incision was opened, and the surrounding connective tissue was removed. Trauma was conducted by dropping a 50 g weight made of 18 mm diameter brass from a height of 1 meter from a plexiglass tube fixed to the stand. The scalp was sutured, and 20 mg/kg resveratrol was orally administered to animals only once. The animal was placed in its normal cage and observed for 24 hours.

Measurements of MDA and GSH content

At the end of the experimental protocol (at the end of the 24th hour), all animals were sacrificed under anesthesia. Malondialdehyde (MDA, #MAK085, Merck, Germany), and Glutathione (GSH, #MAK364, Merck, Germany) colorimetric assays were commercially purchased. Blood samples of each rat were centrifuged at 2000 rpm for 10 min, and the supernatant was collected. Serum plasma of blood samples were further analyzed for MDA and GSH contents according to manufacturer manual. Unit for MDA and GSH was nmol/L.

Histological tissue processing

Cerebral tissues were excised for histological sampling. Hippocampal region was dissected from cerebral samples were further analyzed for histological evaluation. Samples were immersed in zinc-formalin and dehydrated through grading alcohol series and incubated in paraffin wax. 5 µm sections were cut from paraffin blocks and stained for hematoxylin eosin dye and immune staining.

Immunostaining procedure

Hippocampal sections were dewaxed, hydrated in grading alcohol series, and washed in distilled water. 3% hydrogen peroxide (H_2O_2) was dropped on slides to block endogen peroxidase activity. After washing in phosphate buffered saline (PBS), sections were incubated overnight with anti-NFκB primary antibody (catalog no: 51-0500, Thermo Fisher Scientific, Massachusetts, US) at + 4°C. Sections were biotinylated and allowed to react with streptavidin peroxidase solution (Thermo Fischer, US) for 15 minutes. After PBS washing, diaminobenzidine (DAB) chromogen was used as a chromogen to observe color change. The reactions were stopped with PBS solution and sections were counter stained with hematoxylin dye. Slides were mounted and imaged with Zeiss Imager A2 light microscope.

Semi-quantitative Analysis of NFκB immunostaining

The staining intensity of NFκB expression was measured by Image J software (version 1.53, http://imagej.nih.gov/ij). Measurement was calculated by the method of Crowe et al. [19]. Quantification was recorded by analyzing ten fields from each specimen per group. In specimens, brown color stands for positive expression of antibody of interest while blue color represent negative expression of antibody of interest. Signal intensity (expression) from a field was calculated by dividing intensity of antibody of interest to whole area of specimen. A value for staining area/whole area was calculated for each specimen from ten fields. An

average value was measured for groups and analyzed for semi-quantitative immunohistochemistry scoring.

Statistical analysis

Statistical analysis was done using the IBM SPSS 25.0 software (IBM, Armonk, New York, US). Data distribution was done by Shapiro-Wilk test. The data were recorded as median (IQR). The non-parametric Kruskal-Wallis test was used for analyzes between more than two groups, and the post-hoc Dunn test was used due to the small number of animals in the groups. Statistical significance was accepted for $p<0.05$.

RESULTS

Oxidative stress findings

Statistical analysis of malondialdehyde and glutathione were shown in Table 1. MDA content was significantly increased in TBI group compared to sham group. Contrarily, GSH value was statistically decreased in TBI group compared to control group. Upon TBI, resveratrol treatment significantly lowered the MDA content and increased the GSH content in TBI+Resveratrol group compared to TBI group.

Table 1. MDA and GSH content in experimental groups. Resveratrol treatment improved the MDA and GSH content after TBI.

Groups	**MDA**	**GSH**
Sham	4.12. (2.38-6.04)	1.41 (0.98-1.72)
TBI	15.48 (11.28-21.82)	0.45 (0.26-0.96)
TBI+Resveratrol	9.92 (6.72-12.97)	0.88 (0.64-1.01)
Dunn's test	*<0.001	*=0.003
	**0.012	**0.034

Note: Data was presented as median (IQR), *sham vs TBI, **TBI vs TBI+Resveratrol

Histopathological findings

Cerebral sections showing hippocampus was stained with hematoxylin eosin and illustrated in Figure 1. Sham group showed normal neurons and neuroglial cells in hippocampus. Hippocampal integrity was regular with normal vessels (Figure 1a and 1b). In

TBI group, neurons and neuroglial cells in hippocampus were shrunk and degenerated. Number of neurons were lessened. Many neurons lost their nuclei or pyknotic. Integrity of hippocampus was histologically damaged and disintegrated. Edema with vascular dilatations were observed (Figure 1c and 1d). In TBI+Resveratrol group, neuronal regeneration was increased with increased number of neurons in hippocampus. Neuroglial cells were abundant. Vascular dilatation and edema were lessened (Figure 2e and 2f). Administration of resveratrol alleviated the pathologies after TBI.

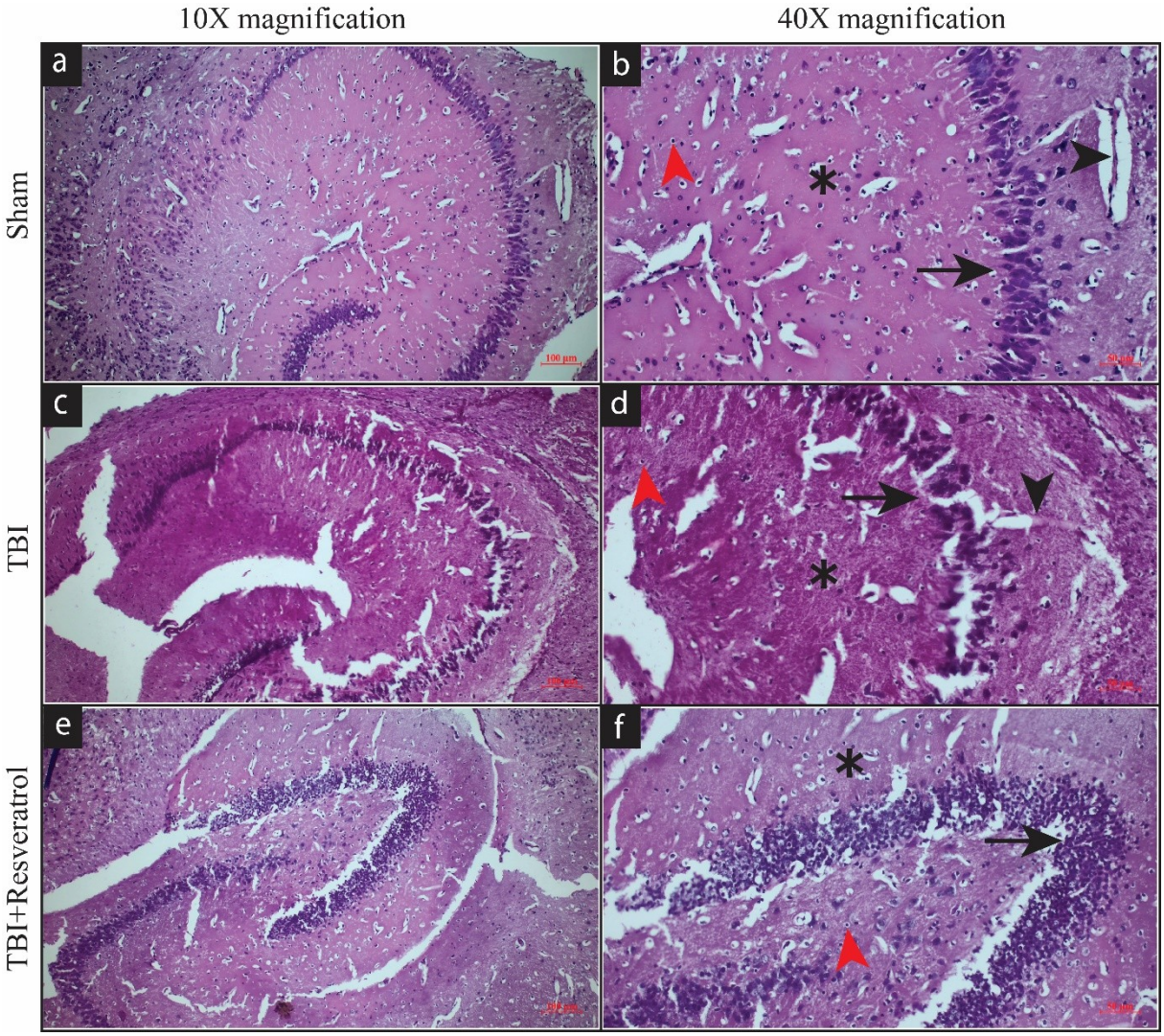

Figure 1. Hematoxylin Eosin staining sections through the hippocampus showing C-shaped hippocampal proper (Cornu ammonis) and dentate gyrus. **1a and 1b:** Neurons (arrow), neuroglia (red arrowhead), vessels (black arrowhead) and hippocampal strata (asterisk) with no pathology in sham group; **1c and 1d:** Degenerated neurons (arrow), neuroglia (red arrowhead), dilated vessels with edema (black arrowhead) and disintegrated hippocampal strata (asterisk) in hippocampus in TBI group; **1e and 1f:** Neuron (arrow) and neuroglial (red

arrowhead) proliferation, and hippocampal strata (asterisk) in TBI+Resveratrol group. **Scale Bar:** 100 µm (10X magnification, total hippocampus), 50 µm (20X magnification).

NFκB immunostaining findings

NFκB immunoreactivity in groups was shown in Figure 2. In sham group, NFκB expression was negative in hippocampal neurons. Hippocampal strata showed negative NFκB immunoexpression. Neuroglial and vascular cells were also negative NFκB expression (Figure 2a and 2b). In TBI group, NFκB level was upregulated in hippocampal neurons, neuroglial cells of hippocampal strata and vascular endothelial cells (2c and 2d). In TBI+Resveratrol group, NFκB immune reactivity was decreased in neurons and neuroglial cells. Hippocampal strata had predominantly negative expression (2e and 2f).

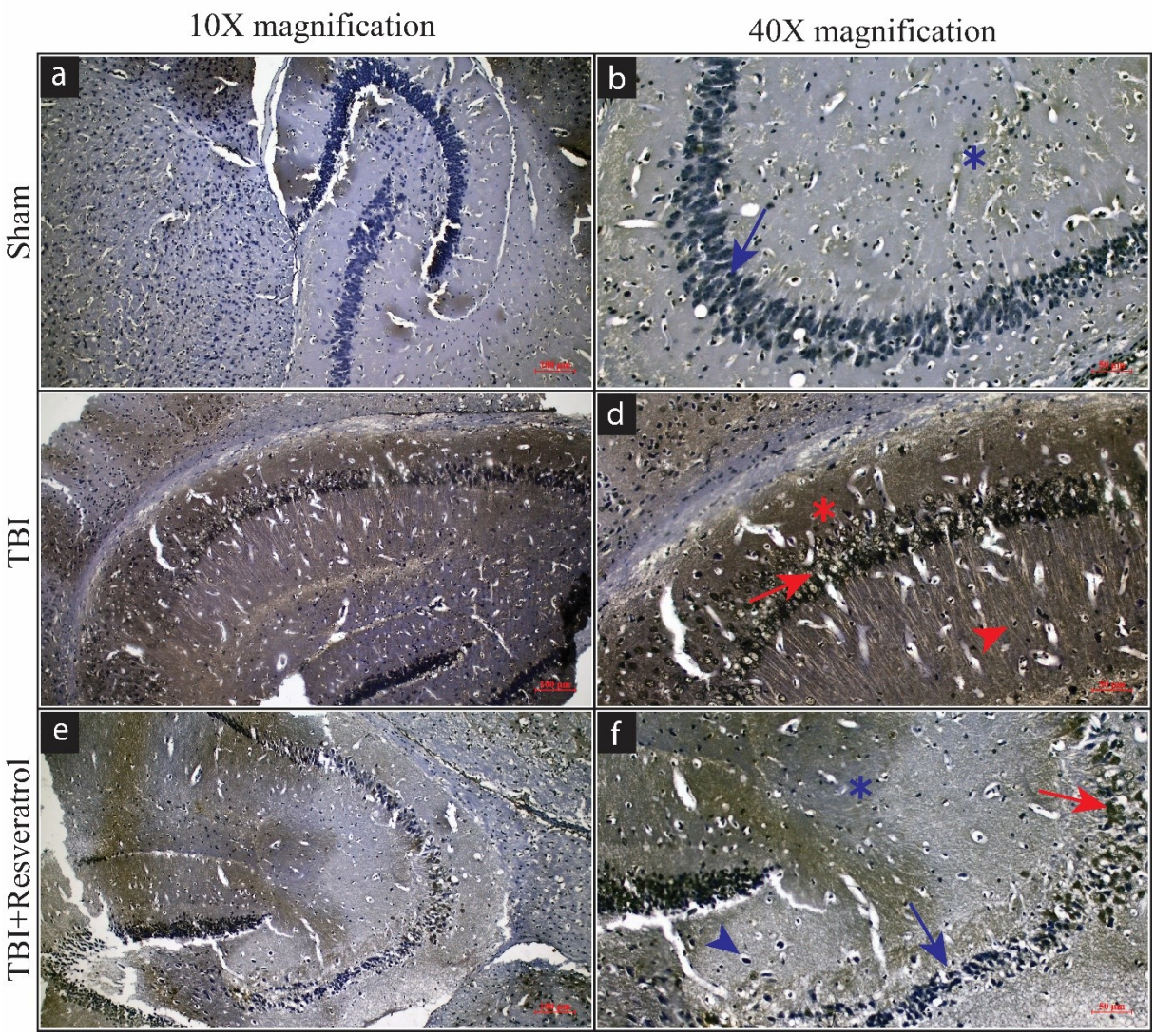

Figure 2. NFκB immunostaining sections through the hippocampus showing C-shaped hippocampal proper (Cornu ammonis) and dentate gyrus. **1a and 1b:** Negative NFκB expression in sham group, neurons (blue arrow) and hippocampal strata (blue asterisk); **1c**

and 1d: Positive immunoreactivity of hippocampal strata (red asterisk) in hippocampus of TBI group; **1e and 1f:** Positive expression of NFκB in neurons (red arrow), negative immunoexpression of NFκB in neurons (blue arrow), in neuroglial cells (blue arrowhead), and in hippocampal strata (asterisk) in hippocampus of TBI+Resveratrol group; **Scale Bar:** 100 µm (10X magnification, total hippocampus), 50 µm (20X magnification).

Expression evaluation: Semi-quantitative measurement of NFκB expression in hippocampus of groups was evaluated and shown in Figure 3. Statistical analysis showed that NFκB was upregulated after TBI however resveratrol treatment reduced its expression.

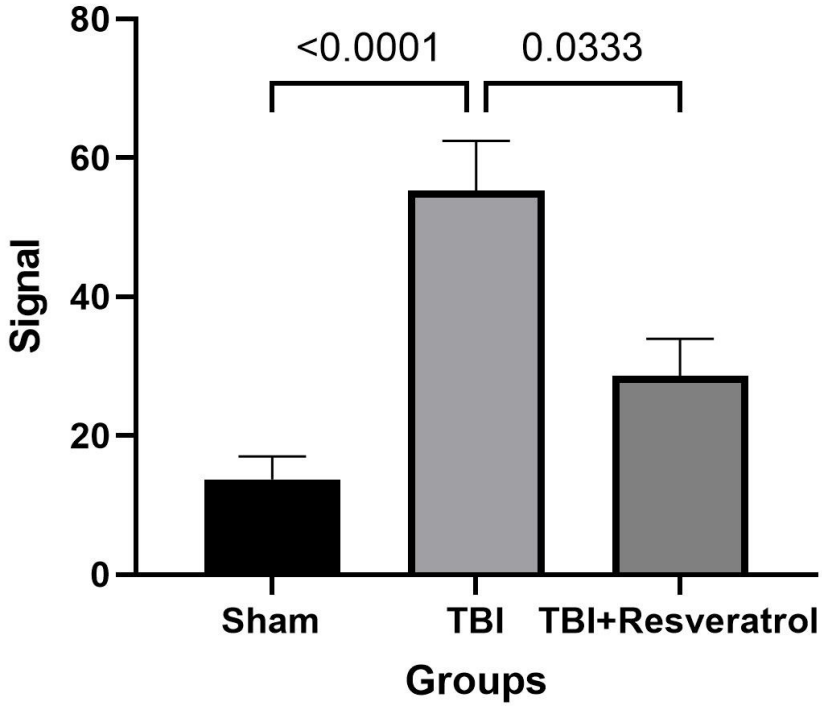

Figure 3. Semi-quantitative analysis of NFκB expression for each groups and binary comparisons of groups.

DISCUSSION

Traumatic brain injury is a global health problem leading to death and disability. After TBI, many molecular cascades are activated following primary or secondary injury. Biochemical cascades generated production of reactive oxygen species (ROS), disrupting balance of oxidant and antioxidant agents in cell [20,21]. For that purpose, medicinal plants with biological activities has been proposed as a treatment method. Özevren et al. studied experimental TBI in rats and found that malondialdehyde content increased and no change in

level of glutathione in trauma group [22]. Similarly, Baloglu et al. found that level of MDA was higher and GSH level were lower in trauma group than in sham group in an experimental TBI model [23]. In another study, TBI-induced inflammation caused excessive ROS production [24]. Biochemical results of this study showed that TBI increased pro-oxidant MDA content and lowered the antioxidant GSH content by disrupting antioxidant defense system of cell and reduced the number of antioxidative agents. Resveratrol with its antioxidant activity acts as free radical scavenger and promoted the antioxidant defensive enzyme production after TBI. Our biochemical results are consistent with previous TBI studies.

Resveratrol is a natural polyphenolic phytoalexin that interferes with the activity of inflammatory and immunoregulatory cytokines and enzymes [25]. Resveratrol suppresses the inflammatory response through the NFκB signaling pathway [26]. Ding et al. studied the impacts of resveratrol on lipopolysaccharide (LPS)-induced inflammation in intestine and found that resveratrol treatment lowered the abundance of bacteria and alleviated the adverse impacts of LPS induced inflammation in intestine. The authors said that resveratrol had preventive role in inflammation [27]. Resveratrol showed immunomodulatory activity by inhibiting the production of interleukin-2, tumor necrosis factor α (TNFα) from lymphocytes [28]. Xian et al. stated that resveratrol treatment reduced the inflammatory mediators and improved the diabetic nephropathy in mice. In this study, TBI caused histopathological alterations in hippocampal strata. Resveratrol treatment improved these pathologies after TBI. Similarly, NFκB immunoreactivity was increased after TBI, showing that TBI induced inflammation via NFκB inflammatory pathway. Resveratrol treatment after TBI, downregulated the NFκB immunoexpression, promoting the neuronal regeneration and survival, protecting the tissue integrity of hippocampal strata. Resveratrol with its anti-inflammatory activity, ameliorates the negative effects of TBI and protected the hippocampus against TBI [29].

Resveratrol has many biological activities. This study specifically focused on antioxidant and anti-inflammatory properties of resveratrol on hippocampus after TBI. We suggest that resveratrol could be used as therapeutic agent in TBI with its great potential of radical scavenging and preventing inflammation. However, more studied with preclinic and clinics are required for efficacy of resveratrol on hippocampus.

Limitations of this study include number of animals used in this study is low. A large-scale animal usage could generate more reliable biochemical and histochemical results. The resveratrol was only treated after TBI, and its effect was analyzed after 24 hours. Acute effects

of resveratrol were shown however long term of resveratrol should also be investigated. The study included only biochemical and histological results, but more experimental data should be conducted to support the hypothesis.

CONCLUSIONS

Resveratrol is rapidly absorbed by the body due to its phytoalexin structure and is involved in metabolic processes. Resveratrol showed a neuroprotective effect in hippocampus and hippocampal tissue with phytoalexin and antioxidant properties by reducing oxidative stress and inflammation in the brain after TBI. In addition, resveratrol treatment also augmented tissue regeneration by promoting cell survival and preventing cell death.

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