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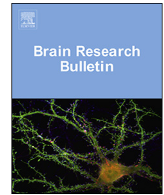

The effect of high mobility group box-1 protein on cerebral edema, blood-brain barrier, oxidative stress and apoptosis in an experimental traumatic brain injury model☆

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ABSTRACT

Traumatic brain injury (TBI) is one of the important reason of morbidity and mortality. While the primary injury due to mechanical impact is unavoidable, the secondary injury which is formed as a result of primary injury and thought to occur due to neuroinflammation in the forefront can be prevented and by this way mortality and morbidity can be reduced. High mobility group box-1 (HMGB1) is a protein that triggers the neuroinflammatory process by being released from the nucleus of necrotic tissues after primary injury.

The aim of this study is to investigate the effects of HMGB1 on its receptors TLR4 and RAGE, cerebral edema, blood-brain barrier, oxidative stress and apoptosis causing secondary damage in an experimental traumatic brain injury model.

Weighing between 280–320 g, 10 to 12 weeks-old, a total of 30 adult male Sprague-Dawley rats were used for the experiments. The rats were randomly assigned to 3 groups: 1) Control, 2) TBI and 3) TBI + ethyl pyruvate group (n = 10 per group). Right parietal cortical contusion was made by using a weight-dropping TBI method. Brain samples were harvested from pericontusional area at 24 h after TBI. HMGB1, TLR4, RAGE, occludin, claudin-5, ZO-1 levels are investigated by western blot analyses and immunohistochemistry examinations. HMGB-1, TLR4 and RAGE expressions increased after TBI. Major tight junction proteins in the blood-brain barrier: occludin, claudin-5 and ZO-1 expressions decreased after TBI. Brain edema increased after TBI. Also, proapoptotic bax and active caspase 3 expressions increased, antiapoptotic bcl-2 levels decreased after TBI. Total oxidant status and oxidative stress increased, total antioxidant status decreased after TBI.

HMGB-1 protein plays a key role in the pathophysiology of traumatic brain injury.

1. Introduction

Traumatic brain injury is one of the most important reason of morbidity and mortality, which causes a significant neurological deficit especially in young adults (Langlois et al., 2006). One or more secondary events in one-third of patients with common head injury emerge within the period between the trauma and the initiation of the patient's treatment, and these events almost double the mortality and morbidity (Chesnut, 1995). In the approach to head injury, the most important point patients who survive until the emergency medical

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intervention is to fight secondary events that follow the initial event and increase brain damage (Walleck, 1992). Extracellular release of various ions, molecules and proteins called damage-associated molecular patterns (DAMPs) from damaged and died cells as a result of primary damage in traumatic brain injury (de Rivero Vaccari et al., 2014). Some of these molecules are ATP, potassium, double-stranded DNA and high mobility group 1 chromatin protein. High mobility group box 1 protein (HMGB1) is a nonhistone DNA binding protein that is involved in the stability and gene transcription of the nucleosomal structure. HMGB1 released in extracellular form from necrotic cells as a result of primary damage in traumatic brain injury, induces inflammation (Scaffidi et al., 2002).

In order to contribute to the pathophysiology of the inflammatory response that causes secondary injury after head trauma, the aim of this study is to investigate the effects of HMGB1 protein on oxidative stress and apoptosis and the effect of HMGB1 protein release on cerebral edema through the level of the tight-junction molecules at the blood-brain barrier in rats with experimental head trauma, which has not been shown in previous studies.

2. Material and methods

2.1. Experimental animals, anesthesia and head trauma model

In this study, totally 30 male adult 10–12-week-old and weighing between 280 and 320 g Spraque-Dawley rats were used, which were not used in any experiment before. During the study, the rats were allowed to freely reach water by feeding with standard pellet rat feed at room temperature (22 ± 2 °C) and 12 h light-12 h dark light cycles and 50–60% humidity environment. Before starting the study, the rats were observed in this environment for one week and their adaptation to the environment was observed. Local Ethic Board of Bezmialem University for Animal Experiments has approved the study.

The experimental animals were administered preoperative anesthesia of 60 mg/kg ketamine hydrochloride (Parke Davis, Istanbul) and 5 mg/kg xylazine hydrochloride (Rompun 2% solution, 50 cc vial, Bayer-Turk Ilac Ltd. Istanbul) during spontaneous respiration through intramuscular route in the back leg. After sedation was ensured, the rats' heads were shaved. The rats were then given the prone position on a rigid sponge in the stereotaxic instrument (World Precision Instruments, Stereotaxic Frame Model No: 502,600 Florida/USA). The head trauma model defined by Feeney et al in 1981 and established by lowering weight on the head (acceleration) was used (Feeney et al., 1981).

2.2. Experiment groups

Rats were randomly selected and three groups were formed each containing 10. Head trauma was applied to the other groups except for the control group.

Group 1 (Control) (n: 10): A right parietal craniotomy was performed and cutaneous-subcutaneous layers were sutured and closed, without applying trauma. Next, 0.9% saline solution was injected at minutes 30, 90, and hour 6 through intraperitoneal route (7.5 ml/kg).

Group 2 (Trauma) (n: 10): A right parietal craniotomy was performed and cutaneous-subcutaneous layers were sutured and closed, applying trauma. Next, 0.9% saline solution was injected at minutes 30, 90, and hour 6 through intraperitoneal route (7.5 ml/kg).

Group 3 (Trauma + ethyl pyruvate) (n: 10): A right parietal craniotomy was performed and cutaneous-subcutaneous layers were sutured and closed, applying trauma. Postoperative 75 mg/kg ethyl pyruvate was injected to the rats through intraperitoneal route from the solution (0.9% saline solution 10 mg/ml) prepared at minutes 30 and 90 and hour 6.

2.3. The sacrification procedure, tissue sampling and calculation of edema volume

After anesthesia was administered to and sedation was ensured for the control group following craniotomy and the other groups at the 24th hour following trauma, blood was drawn 10 cc from all rats through intracardiac route and placed in the dry (w/o gel) biochemistry tubes. The rats were then sacrified via the decapitation process. The brain was extracted from cranium without traumatization. Pericontusional area in the width of 3 mm in peripheral to the contusion area was carefully extracted including cortical and subcortical deep cerebral tissues. The specimens were placed into the tubes containing 10% formaldehyde.

Sampled cerebral tissue remained after extracting the brains of all the rats decapitated 24 h after the operation in all groups, were placed in the cups with known tare weight and weighted via sensitive scales. These tubes were then incubated for 24 h in a Pasteur oven with a fixed temperature of 105 °C. The tubes were reweighed to determine the dry weight. Using the formula of wet weight - Dry weight / wet weight x 100, volume of edema was calculated.

2.4. Immunohistochemical method

For immunohistochemical studies, at the 24th hour after sacrifying the rats, cerebral tissue was extracted completely, the pericontusional area in thickness of 3 mm was separated and fixated in 10% neutral formalin. Following fixation, the tissue samples were embedded into paraffin of 58 °C by processing in a fully-automated tissue monitoring device (Histokinet) (Leica TP 1020 and Leica EG 1160; Leica, Wetzlar, Germany) Sections in thickness of 4-μm obtained from the paraffin blocks using a microtome device (Leica RM2155; Leica, Wetzlar, Germany) were affixed to positively charged slides. HMGB1, TLR4, RAGE, Occludin, Claudin-5 and ZO-1 immunoreactivities were investigated in an immunohistochemical analysis using the Streptavidin-Biotin Peroxidase Complex (StrepABC) method.

2.4.1. Streptavidin-biotin peroxidase complex (StrepABC) method

The paraffin sections were hydrated after deparaffinization and washed with purified water. After washing the positive-charged slides containing the sections in purified water, they were boiled in a microwave oven for 10 min (2 x 5 min) in a citrate buffer (pH 6.0) under 700 W, and then left for cooling at room temperature for 20 min. The slides washed in the purified water after the citrate buffer were waited for 9–10 min (3 × 3 min) in the salted phosphate buffer (PBS; 0.01 M; pH 7.4). In order to prevent forming of endogenous peroxidase activity in 3% hydrogen peroxide (H_2O_2) (Merck, 1.08597) prepared in methanol in room temperature and humid environment, it was kept in a dark environment for 10 min and taken back to the PBS. Then blocking serum was applied to the sections for 10 min in room temperature (Thermo Scientific, TA-125-UB; Deutsch, Germany). In accordance with the antibody application protocol, the sections were incubated including one night (18–19 hours) at +4 °C in the HMGB1 (Novus, NB100-2322), RAGE (Novus, NBP2-03950) and Tight Junction Protein 1 (ZO-1) (Novus, NBP1-85046) antibodies and at 37 °C in an incubator for 1 h in the TLR4 (Novus, NB100-56566), Occludin (Novus, NBP1-87402) and Claudin-5 (Novus, NB120-15106) antibodies. The HMGB1, TLR4, Occludin and ZO-1 antibodies were diluted using the antibody diluent (Thermo Scientific, TA-125-UD) in the ratio of 1:100 and the RAGE and Claudin-5 antibodies were diluted in the ratio of 1:50. After the antibody application, the sections were washed in the PBS for 10 min and incubated then with biotinylated anti-rabbit secondary antibody obtained from goat for 30 min in room temperature (Thermo Scientific, TP-125-BN). After washing the slides in the PBS for 10 min, they were kept in the streptavidin (Thermo Scientific, TS-125-HR) marked with peroxidase for 10 min in room temperature. The sections were incubated with 3-amino-9-ethylcarbazole (AEC) (Thermo

Scientific, TA-004-HAC) in a dark environment for about 10 min based on watching the reaction under microscope for the formation of colored reaction product and then washed in the distilled water to stop the reaction. After washing, the sections were kept in Mayer hematoxylin (Thermo Scientific, TA-125-MH) for 1 min for the opposite staining stage. The sections were washed in the tap water for 5 min and then were covered with water-based concealer (Thermo Scientific, TA-060-UG). Distilled water was applied to the negative control sections instead of primary antibody. The sections were inspected under Leica DM4000 B light microscope and photos were taken.

2.5. Determination of DNA fragmentation (The TUNEL method)

Pericontusional cerebral tissue specimens fixated with formalin were embedded in paraffin at 58°C. Sections in thickness of 4-μm obtained from the paraffin blocks using a microtome device were affixed to positively charged slides. Apoptotic cells in the sections were showed with the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method by using the ApopTag® Plus Peroxidase *in situ* Apoptosis Kit (Chemicon International; Darmstadt, Germany). The sections in thickness of 4 μm obtained from the paraffin blocks were kept at 37 °C for one night. The sections that were deparaffinized by passing through the xylol and reduced alcohol sequences were washed in the PBS. Next, the sections were incubated in the incubator with the 20 μg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) enzyme for 15 min at 37 °C. 3% H_2O_2 (Merck, 1.08597) prepared in the PBS was applied to the sections in a dark environment for 5 min to prevent the endogenous peroxidase activity. TdT enzyme was applied to the sections for 1.5 h, which were kept in the balancing buffer in the incubator for 10 min at 37 °C. In order to stop the reaction of the TdT enzyme, after applying the stopping/washing buffer in the kit for 10 min at room temperature, the sections were washed for 3 min (3 x 1 min) in the PBS. Next, the anti-digoxigenin peroxidase enzyme was applied in the incubator at 37 °C. After washing in the PBS for 8 (4 × 2) minutes, 3.3′-diaminobenzidine (DAB) substrate was applied to the sections as chromogen. After application for 30–45 minutes, the reaction in brown color was stopped with distilled water. After this stage, the sections were stained with methyl green, which was used as the opposite dye, and rapidly passed through butanol. The slides that were made transparent by applying xylol for 15 min (3 x 5 min) in total were covered with ental and prepared to review using a light microscope. During the stage of applying the TdT enzyme, distilled water was used instead of enzyme in the negative control preparation.

2.6. Calculation of the H-Score and apoptotic index

All evaluations were performed independently by two blind observers. The reactions in the tissue sections obtained from pericontusional area following immunohistochemical staining were evaluated under 400x magnification and randomly selected 8–10 different fields for all individuals of 3 different groups. In order to analyze the immunohistochemical HMGB1, TLR4, RAGE, Occludin, Claudin-5 and ZO-1 staining, the immune-positive cells in the sections were evaluated using the histological score (H-Score). The positive stained cells were counted and graded according to the staining intensity: Score were accepted as 0 is negative; 1 is minimal; 2 is moderate; 3 is intense. The H-Score value for each tissue was determined using the formula H-Score = Pi (i + 1). In the formula, “i” indicates the intensity score (degree) and “Pi” indicates the percent value of stained cells. Photos were taken using the Leica DM4000 B microscope (Leica) for all sections.

The apoptotic index was determined independently by two blind observers by counting under 400x magnification and randomly selected 8–10 different fields containing average 800–1000 cells for all individuals of 3 different groups. The results of the observers were compared and the preparations with incompatible results were reviewed. The apoptotic index after counting cells: Was calculated using the formula 100 x (TUNEL-number of positive cell nucleus / number of total cell nucleus).

2.7. The western blot method

For the Western blot analysis, tissues were centrifuged at 14,000 g for 15 min at +4 °C after homogenization. After centrifuge, the supernatants were collected and kept at −20 °C until the time of use. The protein amount of all samples was measured by the Bradford method and the protein amounts were equalized with 10x SDS (sodium dodecyl sulfate) buffer. Samples with equal amounts of protein were then boiled together with the loading buffer at 95 °C for 5 min to allow the proteins to be denatured. Samples were loaded to SDS-Polyacrylamide gel of 12% to ensure 80 μg in each well. Electrophoresis was performed to allow the gel 80 V for 20 min and 120 V for 100 min. After electrophoresis, the samples in the gel were transferred to the PVDF (Polyvinylidene Fluoride) membrane using the IBlot (Invitrogen, U.S.A) device. The membrane was incubated by shaking for 1 h at room temperature using skimmed milk powder prepared as 5% with TBS-T (Tris-base saline-Tween 50 mM Tris, 150 mM NaCl 0.05% Tween 20). After incubation, the membrane was washed with TBS 3 times for 5 min each time. The membranes were cut in accordance with the size predicted by considering the protein marker (Multicolor High Range Protein Ladder 26,625 Thermo Scientific, MA, USA)

These processes were repeated for each antibody. The HMGB1 (1:1000 dilution NB100-2322 Novus Biotechnology, NA, USA), TLR4 (1:1000 dilution, NB100-56566 Novus Biotechnology, NA, USA), RAGE (1:1000 dilution, NBP2-03950 Novus Biotechnology, NA, USA), Occludin (1:1000 NBP1-87402 Novus Biotechnology, NA, USA), Claudine-5 (1:1000 NB120-1510 Novus Biotechnology, NA, USA), ZO-1 (1:1000 dilution, NBP1-85046 Novus Biotechnology, NA, USA), Bax (1:1000 dilution, NB110-55492, Novus Biotechnology, NA, USA) Bcl-2 (1:1000 dilution NB100-92142, Novus Biotechnology, NA, USA), Active Caspase-3 (1:1000 dilution, NB100-56113 Novus Biotechnology, NA, USA) and ß-Actin (1:1000 dilution, NBP1-47423 Novus Biotechnology, NA, USA) antibodies were used in our study. After 24 h, each membrane was washed again with TBS-T 3 times for 5 min each time. The membranes washed with TBS-T were incubated by shaking at room temperature for 1 h with the HRP-conjugated secondary antibody. The membranes were kept at room temperature for 1 min in the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) solution for chemiluminescence imaging. Imaging was performed via the Vilber Laurmat Fx5 Fusion (Vilber Laurmat XA, USA) imaging system. Image *J* (Glyko, Novato, CA, USA) software was used for analysis of the obtained images in optic density.

2.8. Biochemical method

2.8.1. Total antioxidant status (TAS) and total oxidant status (TOS)

TAS and TOS measurements were made and the status of the oxidant and antioxidant agents in the cell of the ethyl pyruvate and head trauma was determined.

This is a fully automated method developed by Erel et al. (Erel, 2004, 2005) to use for measuring the total antioxidant status of the body against strong free radicals. For TAS measurement, the reagent 1 (10 mM o-Dianisidine and 45 AM $Fe(NH_4)_2(SO_4)_2$-$6H_2O$ are solved and prepared in 75 mM Clark buffer (pH = 1.8) and reagent 2 (7.5 mM hydrogen peroxide is mixed and prepared in 75 mM Clark buffer (pH = 1.8) were prepared. 100 μl reagent 1 and 150 μl reagent 2 were included in the 50 μl sample and they were incubated for 10 min at 37 °C and a spectrophotometric measurement was made at 412 nm. For TOS measurement, reagent A (25 mM H_2SO_4 was solved in 140 mM of NaCI solution and the main solution was obtained, first glycerol in the ratio of 10% was solved in the main solution and then 250 μM Xlenol orange was solved and prepared in total volume) and reagent B (10 mM

o-Dianisidine dihydrochloride was solved first in the main solution and then 5 mM ammonia ferrous sulfate was solved and the reagent was prepared) were prepared. 100 µl was taken from each sample and 150 µl of reagent A and 150 µl of reagent B were included in them respectively. They were incubated for 1 min at 37 °C and a spectrophotometric reading was taken at 240 nm. Trolox, a water-soluble analogue of vitamin E, was used as a calibrator and the results were expressed in mmol.Trolox.equivalent/L. The Abbott ARCHITECT i2000 SR autoanalyzer was used for all spectrophotometric measurements.

2.9. Calculation of oxidative stress index (OSI)

The TAS unit was converted into µmol.Trolox.equivalent/L and the oxidative stress index was calculated using the TOS/TAS x100 formula.

2.10. Statistical analysis

The results obtained at the end of the study were calculated as mean ± standard error. After testing the appropriateness of the distribution of the groups to normal distribution, the statistical analysis was conducted using the GraphPad Prism (GraphPad Prism Version 5 Software Program, San Diego, CA) Program, and the Tukey multiple comparisons test after one-way variance analysis (ANOVA). The value $p < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of brain water content

Brain water content was found 77.8% ± 0.2 in the control group. Brain water content was found 80.9% ± 0.5 in the trauma group. The relationship was found statistically significant compared to the control group ($p < 0.05$). It was found that increased brain water content in trauma group regressed to 79.2 ± 0.2% with ethyl pyruvate application and approached control group values (Fig. 1).

3.2. Immunohistochemical findings

The HMGB1, TLR4, RAGE, Occludin, Claudin-5 and ZO-1 levels were evaluated during the immunohistochemical analysis in the pericontusional cerebral tissue sections. Distilled water was applied to the negative control sections with no reaction, instead of primary antibody.

The HMGB1 (Fig. 2), TLR4 (Fig. 3) and RAGE (Fig. 4) levels were evaluated during the immunohistochemical analysis in the pericontusional cerebral tissue sections. The HMGB1 ($p < 0.001$), TLR4 ($p < 0.001$) and RAGE ($p < 0.001$) levels were determined significantly increased in the trauma group when compared to the control group. Notwithstanding, it was observed that the HMGB1 ($p < 0.001$), TLR4 ($p < 0.001$) and RAGE ($p < 0.001$) levels were significantly decreased in the Trauma + EP group with ethyl pyruvate application when compared to the trauma group (Fig. 5). Mean ± standard deviation values of the H-Score values of the HMGB1, TLR4 and RAGE levels were specified in Table 1.

When evaluating the Occludin (Fig. 6), Claudin-5 (Fig. 7) and ZO-1 (Fig. 8) levels during the immunohistochemical analysis of the pericontusional cerebral tissue, it was seen that the Occludin ($p < 0.001$), Claudin-5 ($p < 0.001$) and ZO-1 ($p < 0.001$) levels of the trauma group were significantly decreased when compared to the control group. For the Trauma + EP group, it was found out that the Occludin ($p < 0.001$), Claudin-5 ($p < 0.001$) and ZO-1 ($p < 0.001$) levels were significantly increased when compared to the trauma group (Fig. 9). These results suggest that traumatic brain injury reduces the expression of proteins associated with tight binding. Mean ± standard deviation values of the H-Score values of the Occludin, Claudin-5 and ZO-1 levels were specified in Table 2.

3.3. Findings of DNA fragmentation (TUNEL method)

Apoptotic cells were investigated by the TUNEL method in the sections of pericontusional cerebral tissue.

According to this, apoptotic cells were found in less amount in the control group (Fig. 10). Apoptotic index was found 23.3% ± 0.5 in the trauma group, 3.4% ± 0.3 in the control group and 16.3% ± 0.6 in the trauma + EP group. It was seen that the apoptotic index of the trauma group in the pericontusional area was significantly increased ($p < 0.001$) and the apoptotic index of the trauma + EP group in the same area was significantly decreased ($p < 0.001$) when compared to the trauma group (Fig. 11).

3.4. Findings of western blot method

HMGB1 protein was detected 1.44 ± 0.13 in the trauma group, 0.49 ± 0.09 in the control group and 0.91 ± 0.08 in the trauma + EP group. In the post-traumatic samples, it was seen that this protein level was increased significantly ($p < 0.001$) when compared to the control group, and the expression of HMGB1 protein of the rats with ethyl pyruvate was decreased significantly decreased ($p < 0.01$) when compared to the trauma group (Fig. 12).

The expression of TLR 4 was detected 1.32 ± 0.07 in the trauma group, 0.52 ± 0.04 in the control group and 1.04 ± 0.08 in the trauma + EP group. The expression of TLR4 was found significantly high ($p < 0.001$), similar to the HMGB1 protein. In the Trauma + EP group, the expression of TLR 4 was found significantly low ($p < 0.001$) when compared to the trauma group as a result of inhibition of HMGB1 protein with EP (Fig. 13).

RAGE protein, receptor of HMGB1 protein, was detected 1.26 ± 0.04 in the trauma group, 0.40 ± 0.04 in the control group and 1.27 ± 0.04 in the trauma + EP group. When compared to the control group, RAGE protein was found significantly high ($p < 0.001$) in the animals included in the trauma group. Even though ethyl pyruvate administration partially reduced expression of this receptor compared to the trauma group, this decrease was not found statistically significant (Fig. 14).

Mean ± standard deviation values of the Western Blot values of the HMGB1, TLR4 and RAGE levels were specified in Table 3.

While expressions of the Occludin and Claudin-5 proteins were found 0.52 ± 0.08 and 0.44 ± 0.10, in the trauma group, they were found 1.47 ± 0.16 and 1.61 ± 016 in the control group respectively. A statistically significant relationship was found between the two groups ($p < 0.001$). An increase toward the value in the control group was observed in the Trauma + EP group both in the expressions of

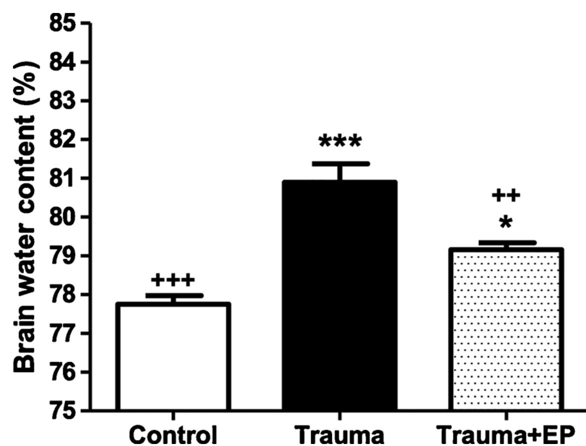


Fig. 1. Changes in the water content of brain in the control, trauma and trauma + EP groups. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. *P < 0.05, ***P < 0.001, compared to control group; ++P < 0.01, +++P < 0.001, significance value compared to trauma group.

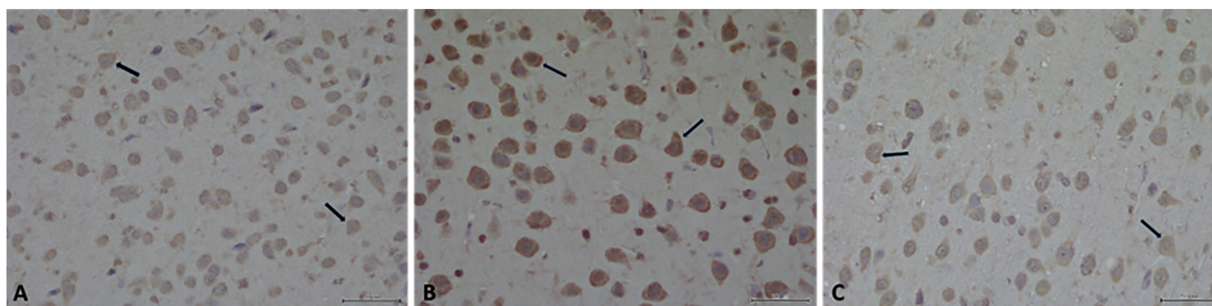


Fig. 2. Immunohistochemical designation of HMGB1 protein levels observed in cytoplasm in pericontusional cerebral tissue samples (indicated with ↑). A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. Bar: 50 µm.

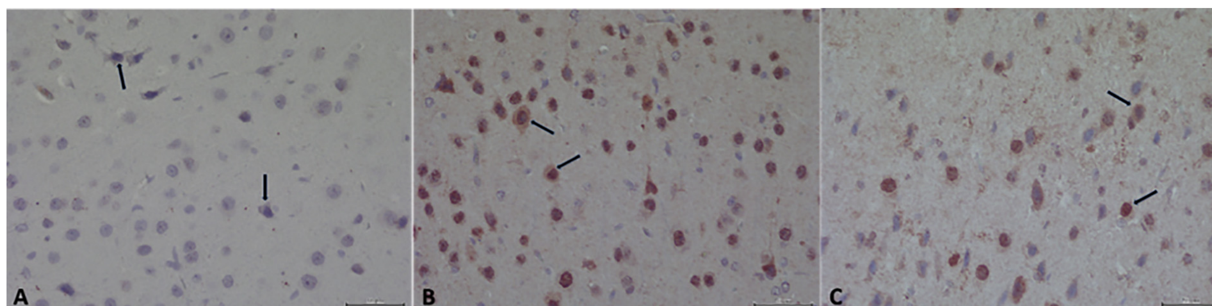


Fig. 3. Immunohistochemical designation of TLR4 levels in pericontusional cerebral tissue samples (indicated with ↑). A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. Bar: 50 µm.

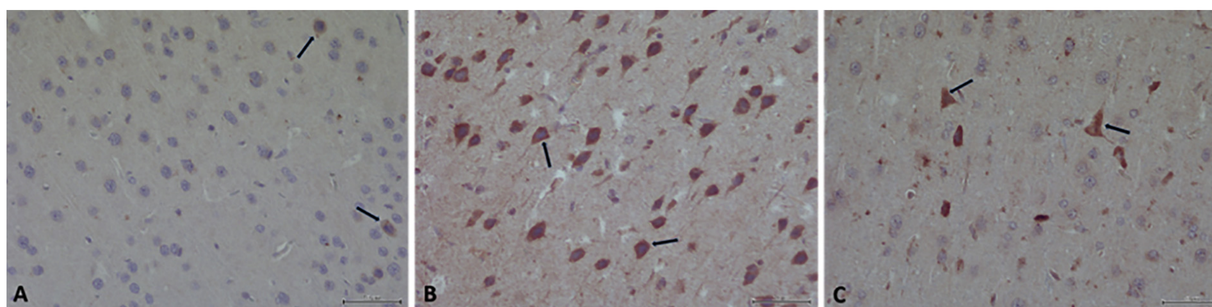


Fig. 4. Immunohistochemical designation of RAGE levels in pericontusional cerebral tissue samples (indicated with ↑). A: Control; B: Trauma; C: Trauma + Ethyl Pyruvate (EP) group. Bar: 50 µm.

Occludin ($p < 0.05$) and Claudin-5 ($p < 0.05$) (Fig. 15 and 16). ZO-1 value was found 1.70 ± 0.08 in the control group and 0.53 ± 0.08 in the trauma group. The findings suggest that the ZO-1 expression is significantly decreased in the trauma group ($p < 0.001$). Even though ethyl pyruvate increased the expression of this protein with HMGB1 inhibition, this increase was not found statistically significant (Fig. 17). Mean ± standard deviation values of the Western Blot values of the Occludin, Claudin-5 and ZO-1 levels were specified in Table 4.

In the study, Bax protein found 0.46 ± 0.06 in the control group was found 1.57 ± 0.13 in the trauma group. It was found significantly high in the trauma group when compared to the control group ($p < 0.001$). This suggested that post-traumatic apoptosis has increased secondary to Bax. As a result of EP application to the rats with trauma, the Bax levels were demonstrated a significant decrease when compared to the trauma group ($p < 0.001$) (Fig. 18). This suggests that HMGB1 inhibition reduces the Bax expression, and consequently inhibition of apoptosis.

Bcl-2 protein was found 1.78 ± 0.04 in the control group and 0.38 ± 0.04 in the trauma group and expression of this protein was considered statistically significant ($p < 0.001$). Bcl-2 protein level was found significantly high as 0.83 ± 0.07 in the post-traumatic EP group secondary to decreased apoptosis ($p < 0.001$) (Fig. 19).

Active Caspase-3 expressions were measured 0.36 ± 0.06 and 1.59 ± 0.10 in the control and trauma groups respectively. Increased active caspase-3 expression in the trauma group was found statistically significant. Excessively expressed active caspase-3 protein suggests increased apoptosis. Active caspase-3 expression was found 0.96 ± 0.07 in the trauma + EP group, which is significantly decreased ($p < 0.001$), and become close to the values in the control group (Fig. 20).

Mean ± standard deviation values of the Western Blot values of the Bax, Bcl-2 and Active Caspase-3 levels are specified in Table 5.

3.5. Biochemical findings

In the study, it was found out that, when the total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) values in the pericontusional cerebral tissue samples, the TAS values were statistically significant decreased ($p < 0.001$) and the TOS ($p < 0.001$) and OSI ($p < 0.001$) values were significantly increased. In the Trauma + EP group, it was seen that the TAS values which were low in the trauma group have increased ($p < 0.001$) and the TOS ($p < 0.001$) and OSI ($p < 0.001$) have decreased, and become close to the values of the control group (Fig. 21). The mean ± standard

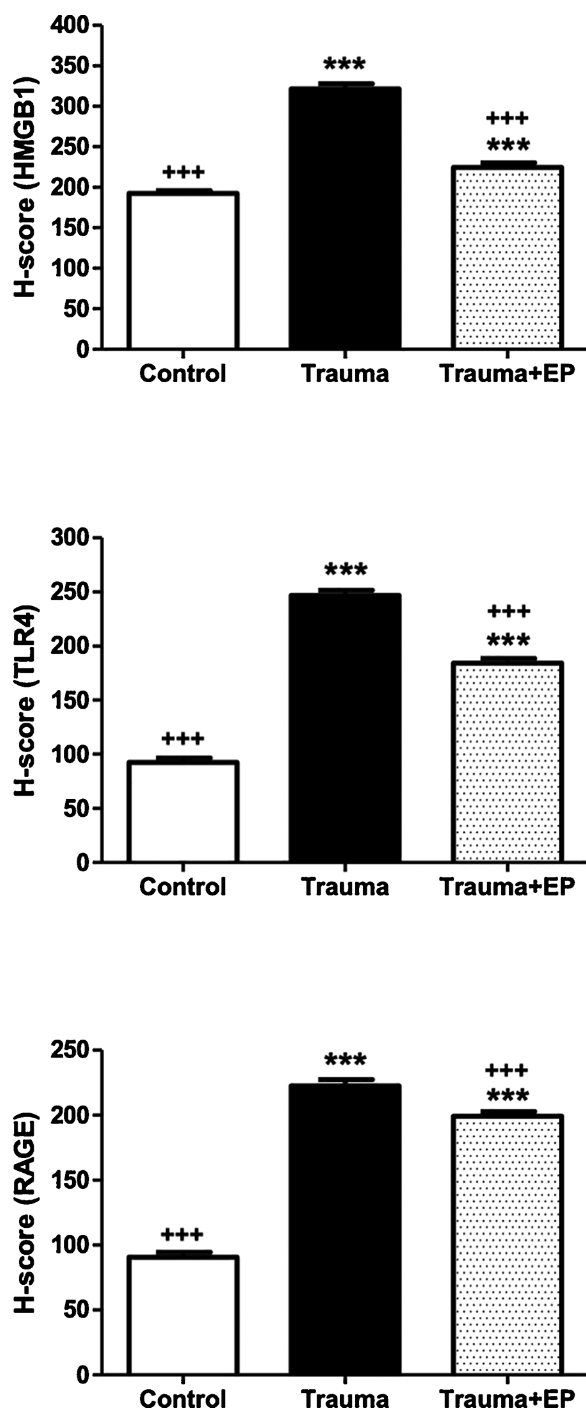


Fig. 5. HMGB1, TLR4 and RAGE levels in pericontusional cerebral tissue samples of all groups. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. ***P < 0.001, compared to control group; + + +P < 0.001, Significance value compared to trauma group.

Table 1
Mean ± standard deviation values of HMGB1, TLR4 and RAGE.

	Control	Trauma	Travuma + EP
HMGB1 (H-Score)	1925 ± 3,4	321,3 ± 6,6	224,6 ± 5,6
TLR4 (H-Score)	9238 ± 4,0	246,9 ± 4,7	184,3 ± 4,3
RAGE (H-Score)	9071 ± 3,8	222,6 ± 4,8	199,2 ± 3,7

deviation values of the total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) are specified in Table 6.

4. Discussion

Secondary damage mechanisms in the pathophysiology of traumatic brain injury have led the researches focus on this direction to prevent catastrophic consequences such as permanent disability and death. Studies conducted for this purpose have shown that neuroinflammation has a role in both acute and chronic stages of traumatic brain injury, and this second mechanism of damage, which leads to extra cell death, may have a key role in the pathology and treatment of head trauma; thus, traumatic brain injury has recently been considered a neuroinflammatory disease of the central nervous system (Acosta et al., 2014; Hernandez-Ontiveros et al., 2013; Niyonkuru et al., 2013; Tajiri et al., 2014; Yu et al., 2009). In the studies, after the pathologies causing neural tissue damage such as trauma and ischemia, it was thought that HMGB1 protein released from the nuclei of necrotic tissues to cytoplasm or extracellular space may be the first step of inflammation by inducing proinflammatory cytokine release. In our study, it was shown that the HMGB1 protein increase inducing the post-traumatic inflammatory response leads to increase in the TLR4 and RAGE levels and progressed cerebral edema by causing decrease of the occludin, claudin-5 and Zo-1, which are tight linking molecules in the blood-brain barrier. It was also found that HMGB1 protein increased apoptosis by increasing bax and active caspase-3 levels and decreasing bcl-2 levels and increased oxidative damage by increasing total oxidative status. It was seen that, for the group with ethyl pyruvate, these effects are in levels similar with the group with no trauma because of HMGB1 inhibition.

4.1. HMGB1 and secondary damage

HMGB1 protein induces the formation steps of events such as blood-brain barrier damage, cerebral edema and brain damage and plays a role in the secondary mechanisms of damage in traumatic brain injury (Shlosberg et al., 2010; Weiss et al., 2009). HMGB1 protein, localized in the nucleus in normal tissue, has been shown to induce the inflammatory response by localizing out of the cytoplasm and extracellular space in post-traumatic necrotic cells (Scaffidi et al., 2002). It was seen that HMGB1 protein caused neural cell death depending on the release of proinflammatory cytokines, by increasing microglial activation in Parkinson's disease (Gao et al., 2011). This activation has become a current issue also in traumatic brain injury that has similar characteristics with neurodegenerative diseases, and in a study, it was found that the post-traumatic increased HMGB1 protein induced microglial activation and thus increased cerebral edema by increasing the expression of Aquaporin 4 that is among astrocytes in the blood-brain barrier (Laird et al., 2014; Kang et al., 2014; Ohnishi et al., 2014). Expression of proinflammatory cytokine (TNF-alpha, IL-1) increases due to microglial activation in post-traumatic cerebral tissue (Szmydynger-Chodobska et al., 2009; Kinoshita et al., 2002). Thus, neuronal damage, called TNF-alpha-induced neurodegeneration, emerges through caspase pathways and microglial glutamate release (Ye et al., 2013). Although it has been argued that proinflammatory cytokines may increase the permeability of the blood-brain barrier by decreasing the expression of occludin, some researchers have argued that their main task is to provide chemokine synthesis by endothelial and astrocytic induction and to increase the migration of inflammatory cells from the blood to the cerebral parenchyma (Mankertz et al., 2000; Szmydynger-Chodobska et al., 2010). In a previous study, it has been found that cerebral edema regressed in rats with anti-HMGB1 antibody due to decrease in blood-brain barrier permeability, MMP-2/9 activity and in TNF-alpha and iNOS expression (Okuma et al., 2012). In a study by Yang et al., matrix metalloproteinases have been shown to damage the basal lamina proteins and tight junction complex (Yang et al., 2007). It has been shown in experimental animal models, that,

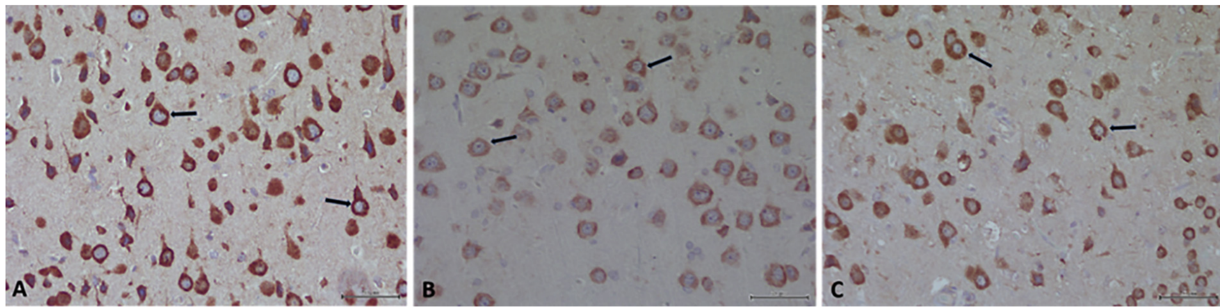


Fig. 6. Immunohistochemical designation of Occludin levels in pericontusional cerebral tissue samples (indicated with ↑). A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. Bar: 50 µm.

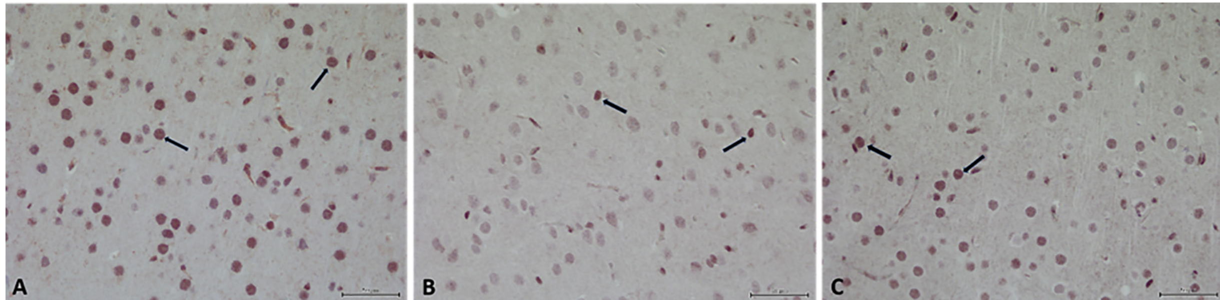


Fig. 7. Immunohistochemical designation of Claudine-5 levels in pericontusional cerebral tissue samples (indicated with ↑). A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. Bar: 50 µm.

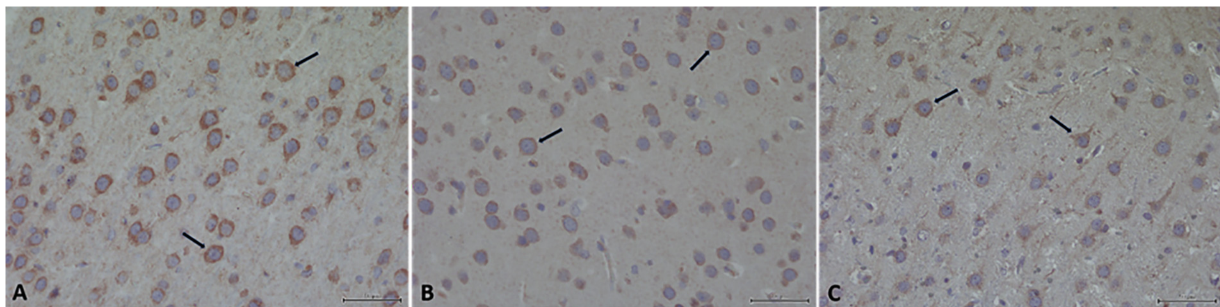


Fig. 8. Immunohistochemical designation of ZO-1 levels in pericontusional cerebral tissue samples (indicated with ↑). A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. Bar: 50 µm.

depending on the increase in IL-1 beta, the tight linking proteins in the blood-brain barrier have been destroyed, thereby leading to a decrease in the levels of occludin and ZO-1, and thus causing an increase in the blood-brain barrier permeability (Bolton et al., 1998). In a study conducted by creating an experimental ischemia model in rats, it has been shown in the electron microscope that the increased HMGB1 protein caused deformation on the blood-brain barrier in tight binding proteins and improvement has been seen in appearance of these structures of the rats with anti-HMGB1 antibody (Zhang et al., 2011). Based on the literature we investigated, it was found out in our study that increased HMGB1 protein levels in the pericontusional cortical and subcortical cerebral tissue in peripheral to the traumatic area in post-traumatic rats caused in a statistically significant decrease in the Occludin, Claudin-5 and ZO-1 levels, which are the tight binding proteins of the blood-brain barrier, and an increase in cerebral edema. In the rats with post-traumatic ethyl pyruvate, HMGB1, Occludin, Claudin-5 and ZO-1 levels and cerebral edema decreased significantly compared to the trauma group as a result of HMGB1 inhibition.

4.2. HMGB1 and apoptosis

Apoptosis is induced that causes destruction of DNA by caspase-3 activation as a result of binding of death receptors (FasL, TNFR, DR5) to the death receptors (FasL, TNFR, DR5) on the cell surface and different pathways such as cytochrome-c released from the mitochondrial inner membrane to the cytosol (Adams and Cory, 2001; Spierings et al., 2004; Curtin and Cotter, 2003; Lewén et al., 2001). When DNA damage occurs in the cell for any reason, if the cell damage is not repaired, the p53 gene activates the bax protein (pro-apoptotic) and allows the cell to die by apoptosis through mitochondria. In a cell, the intracellular bax/bcl-2 ratio is extremely important in deciding whether the cell will go apoptosis. If bax is too much, the cell will go apoptosis; if bcl-2 (anti-apoptotic gene) is too much, apoptosis will be inhibited. There are recent studies suggesting that p53-dependent apoptosis mechanism is responsible for neural cell death after head trauma (Rachmany et al., 2013; Yang et al., 2015). Although there are several studies investigating the effects of HMGB1 protein on apoptosis in spinal cord injury and ischemia-reperfusion injury, there are few studies on traumatic brain injury in the literature (gong et al., 2014; Kawabata et al., 2010. Studies in less number conducted on the subject of traumatic brain injury have shown that HMGB1 protein is associated with cytochrome-c increase (Au et al., 2012). In vitro stage of a study conducted by establishing a cerebral ischemia model in rats suggested that recombinant HMGB1 protein induces apoptosis by increasing the bax/bcl-

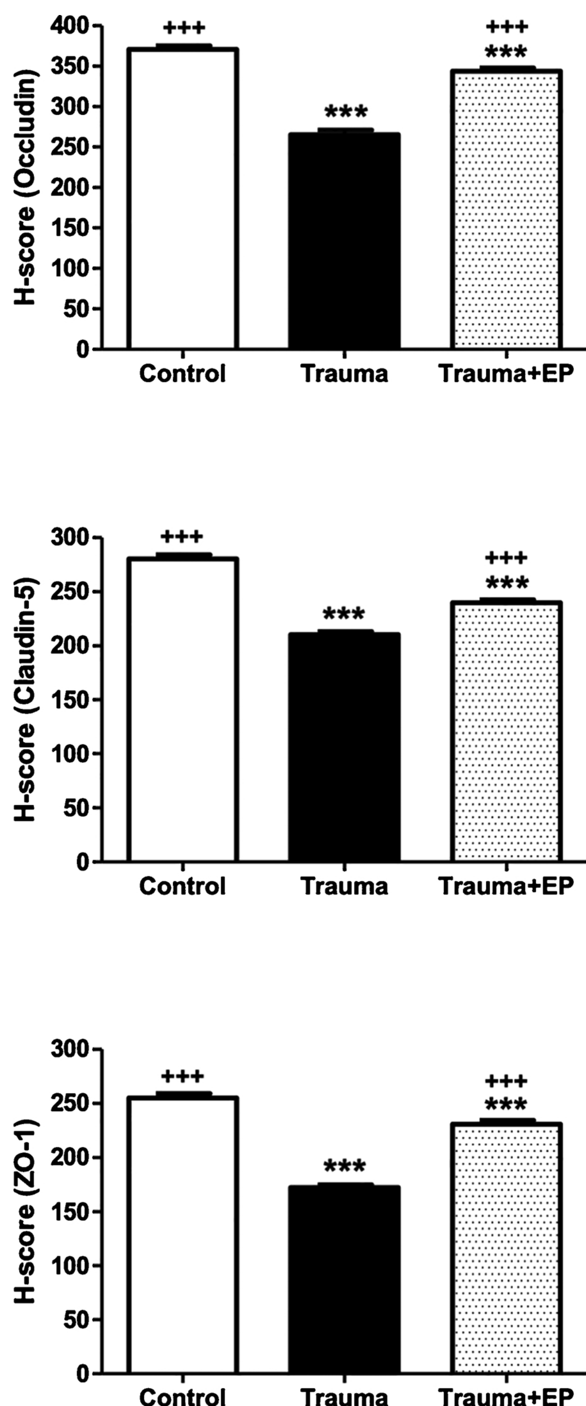


Fig. 9. Occludin, Claudine-5 and ZO-1 levels in pericontusional cerebral tissue samples of all groups. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. ***P < 0.001, compared to control group; + + +P < 0.01, significance value compared to trauma group.

Table 2
H-Score values of Occludin, Claudin-5, ZO-1.

	Control	Trauma	Trauma + EP
Occludin (H-Score)	3709 ± 4,3	265,5 ± 5,5	344,0 ± 3,9
Claudin-5 (H-Score)	2804 ± 3,6	210,3 ± 2,8	239,9 ± 2,8
ZO-1 (H-Score)	2551 ± 4,2	172,4 ± 2,6	231,0 ± 3,4

2 ratio in C6 cells (Ha et al., 2012). Studies conducted by creating a myocardial cerebral ischemic injury in rats suggested that HMGB1 protein regulates the apoptosis mechanisms through molecules such as bax, bcl-2 and caspase-3 (Zhang et al., 2014; Zhai et al., 2012). In addition, in a study conducted by establishing a subarachnoid hemorrhage model in rats, it has been shown that HMGB1 protein increased the caspase-3 levels (Sun et al., 2014). In our study, the apoptotic index was increased significantly compared to the control group in line with the HMGB1 protein increase in the trauma group when tissue samples of the pericontusional area are reviewed. In the Trauma + EP group, the apoptotic index in the same area was observed significantly decreased depending on decrease of HMGB1 protein when compared to the trauma group. The effect of HMGB1 protein on the bax and bcl-2 was also investigated and, depending on the post-traumatic increase of HMGB1 protein, a statistically increase in Bax protein and decrease in the bcl-2 level were seen. In the group with HMGB1 inhibition after administering post-traumatic EP, increase in the bcl-2 level and decrease in the bax level were found compared to the trauma group. When the caspase-3 level was evaluated in the samples obtained from pericontusional tissue, it was found that the expression of caspase-3 increased depending on the increase in HMGB1 level in the trauma group. In the group with HMGB1 inhibition after administering post-traumatic EP, statistically significant decrease in the caspase-3 levels was found compared to the trauma group.

4.3. HMGB1 and receptors

The HMGB1 protein, a DAMP prototype, shows its inflammatory effect by binding to various receptors. These receptors are: RAGE, TLR2 and TLR4 (Muhammad et al., 2008; Yu et al., 2006). Toll-like receptors recognize certain hazard signals such as PAMP and DAMP in cases such as cellular injury and infection and activate the immune system for defense purpose (Akira and Takeda, 2004). A number of studies show that, in cases of HMGB1-induced inflammation, damage, and immunity, the HMGB1 protein has to bind to RAGE receptors to demonstrate its effect. In addition to this, extracellular HMGB1 can stimulate the expression of RAGE in various cell types (Li et al., 1998). Because of HMGB1 receptor interaction, NF−KB is activated and it increases the release of proinflammatory cytokine (Luan et al., 2010; Taguchi et al., 2000; Su et al., 2011). Several studies have been conducted to demonstrate which receptor is more responsible for the effects of HMGB1 protein. In a study conducted by Okuma et al. on this subject by creating a model of traumatic brain injury in rats, they suggested that RAGE receptors are more responsible than TLR5 and TLR2 receptors for increased permeability of the blood-brain barrier caused by the HMGB1 protein (Okuma et al., 2014). On the other hand, in other studies by creating a model of traumatic brain injury, it was seen that the HMGB1 protein shows its effect by binding to the TLR4 receptor, but no comparison with the RAGE receptor was made in these studies (Laird et al., 2014; Su et al., 2011). In a cerebral ischemia study, it was observed that the HMGB1 protein secreted from dead cells bound to the TLR4 receptor, increased the MMP 9 level, and resulting in an increase in ischemic brain injury and neurovascular damage (Qiu et al., 2010). The effect of HMGB1 protein increase on the expressions of TLR4 and RAGE in traumatic brain injury was investigated in our study. It was found that the HMGB1, TLR4 and RAGE levels increased significantly in the trauma group compared to the control group, on the other hand, HMGB1 and TLR4 levels were significantly decreased in the Trauma + EP group with HMGB1 inhibition through ethyl pyruvate administration when compared to the trauma group, and that even though the expression of RAGE was partially decreased compared to the trauma group, this decrease is not statistically significant. As a result, it was concluded that HMGB1 protein was more effective in the expression of TLR4.

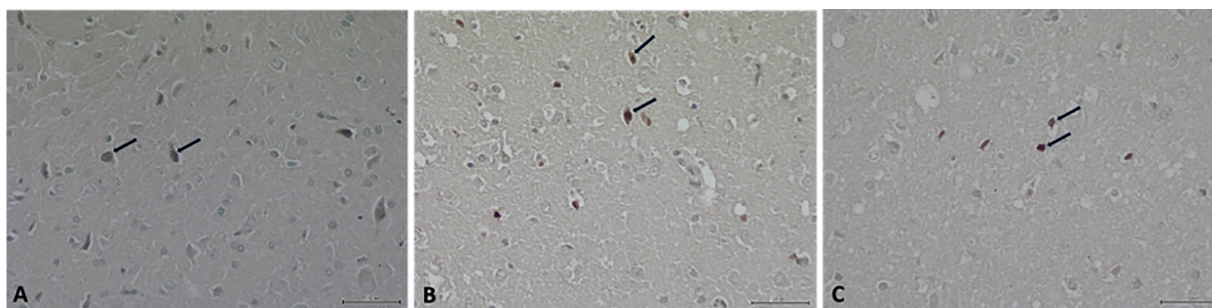


Fig. 10. TUNEL reaction in pericontusional cerebral tissue sections (indicated with ↑). A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. Bar: 50 μm.

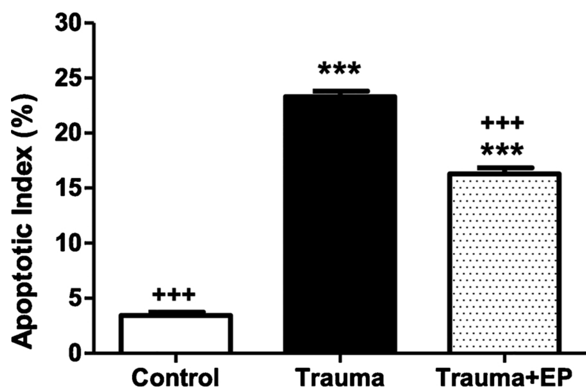


Fig. 11. Comparison of apoptotic indices of experimental groups. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. ***P < 0.001, compared to control group; + + +P < 0.01, significance value compared to trauma group.

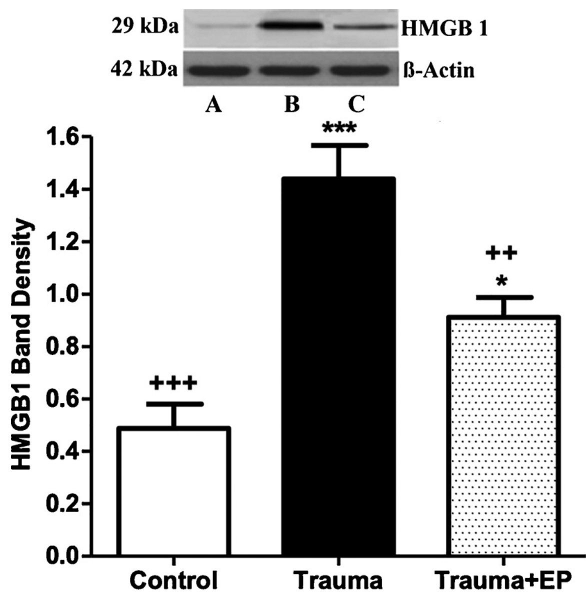


Fig. 12. Designation of the expression of HMGB1 protein by Western Blot method in pericontusional cerebral tissue samples. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. *P < 0.05, ***P < 0.001, compared to control group; + +P < 0.01, + + +P < 0.001, significance value compared to trauma group.

4.4. HMGB1 and oxidative stress

There are many studies about oxidative stress increase in traumatic brain injury (Angeloni et al., 2015; Mojtahedzadeh et al., 2014). Oxidative damage causes post-traumatic neuronal death. Reactive oxygen species in high amount are produced due to stimulation of

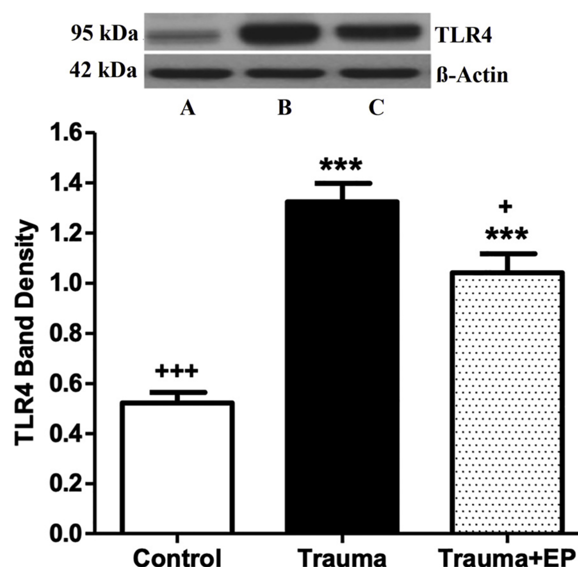


Fig. 13. Designation of the TLR4 expression by Western Blot method in pericontusional cerebral tissue samples A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. ***P < 0.001, compared to control group; +P < 0.05, + + +P < 0.001, significance value compared to trauma group.

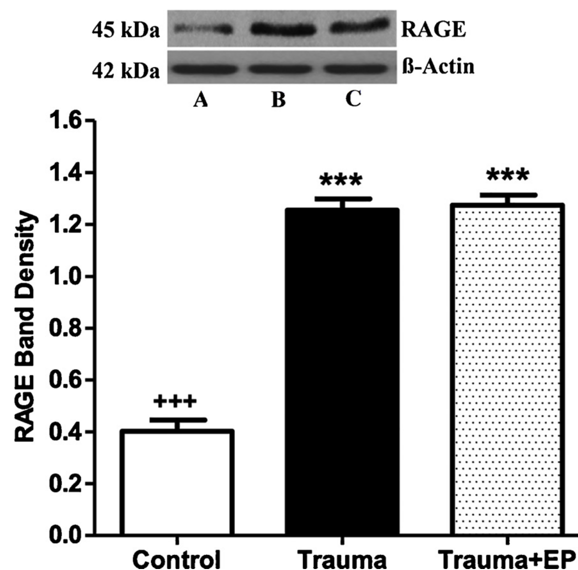


Fig. 14. Designation of the RAGE expression by Western Blot method in pericontusional cerebral tissue samples. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. ***P < 0.001, compared to control group; + + +P < 0.01, significance value compared to trauma group.

Table 3
Western blot values of HMGB1, TLR4 and RAGE.

	Control	Trauma	Trauma + EP
HMGB1/ß-actin	0,49 ± 0,09	1,44 ± 013	0,91 ± 008
TLR4/ß-actin	052 ± 004	1,32 ± 0,07	1,04 ± 008
RAGE/ß-actin	0,40 ± 004	1,26 ± 004	1,27 ± 004

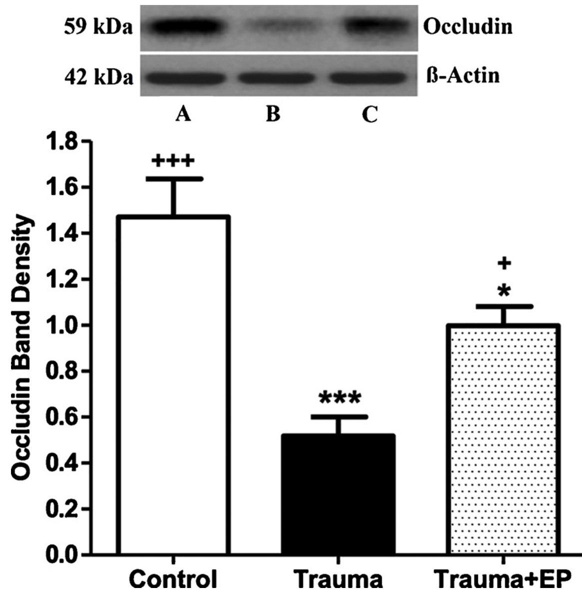


Fig. 15. Designation of the expression of Occludin protein by Western Blot method in pericontusional cerebral tissue samples. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. *P < 0.05, ***P < 0.001, compared to control group; +P < 0.05, + + +P < 0.001, significance value compared to trauma group.

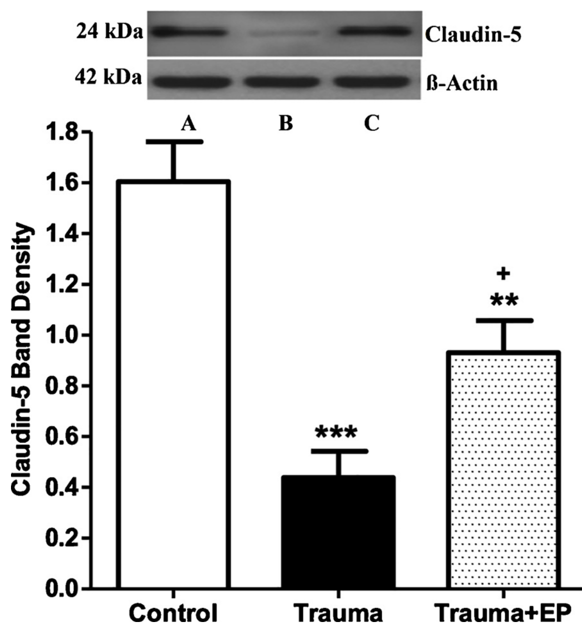


Fig. 16. Designation of the expression of Claudine-5 protein by Western Blot method in pericontusional cerebral tissue samples. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. **P < 0.01, ***P < 0.001, compared to control group; +P < 0.05, + + +P < 0.001, significance value compared to trauma group.

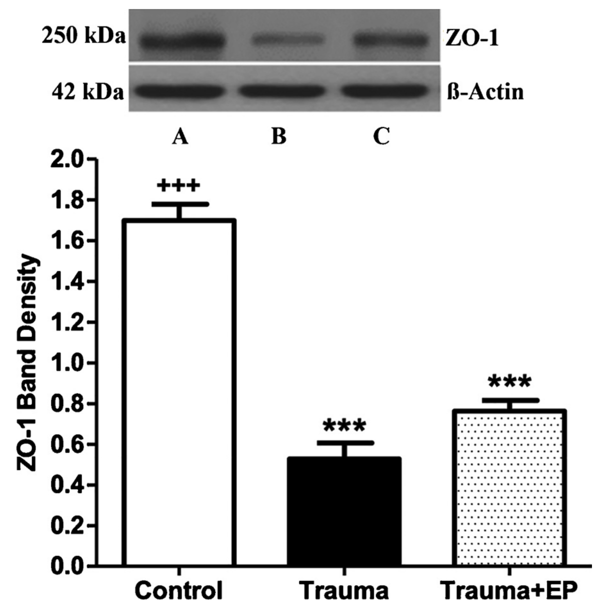


Fig. 17. Designation of the expression of ZO-1 protein by Western Blot method in pericontusional cerebral tissue samples. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. ***P < 0.001, compared to control group; + + +P < 0.01, significance value compared to trauma group.

Table 4
Western blot values of Occludin, Claudin-5 and ZO-1.

	Control	Trauma	Trauma + EP
Occludin/ß-Actin	147 ± 0,16	052 ± 008	1,00 ± 008
Claudin-5/ß-Actin	1,61 ± 016	0,44 ± 010	0,93 ± 013
ZO-1/ß-Actin	170 ± 008	0,53 ± 008	0,76 ± 0,05

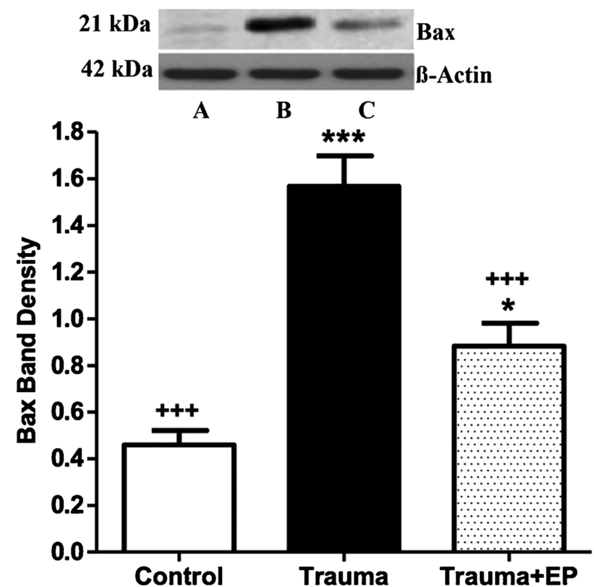


Fig. 18. Designation of the expression of proapoptotic Bax protein by Western Blot method in pericontusional cerebral tissue samples. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. *P < 0.05, ***P < 0.001, compared to control group; + + +P < 0.001, significance value compared to trauma group.

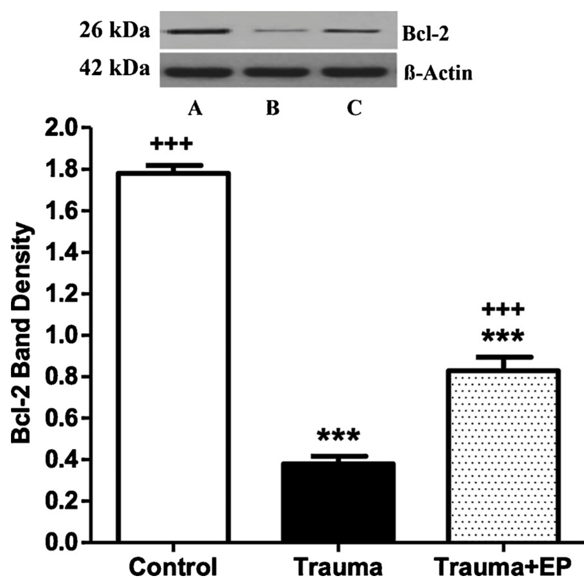


Fig. 19. Designation of the expression of Bcl-2 protein by Western Blot method in pericontusional cerebral tissue samples. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. ***P < 0.001, compared to control group; + + +P < 0.01, significance value compared to trauma group.

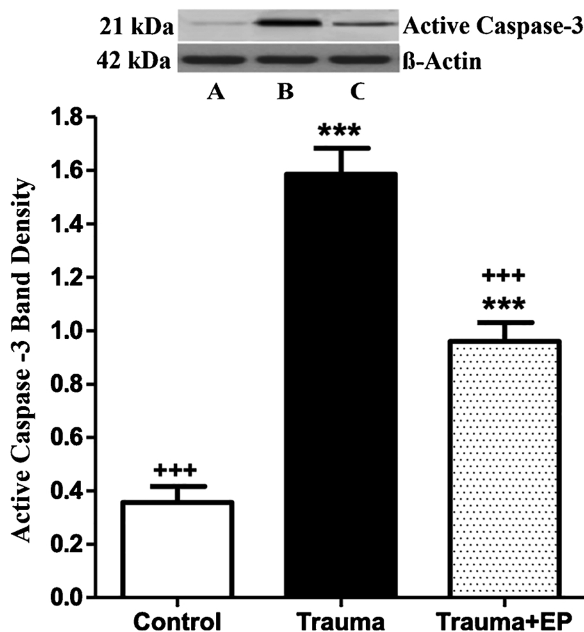
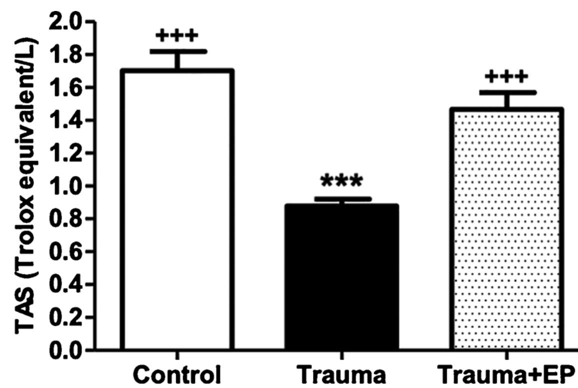
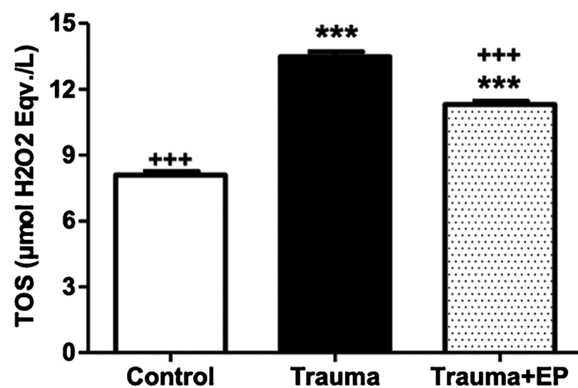


Fig. 20. Designation of the expression of active caspase-3 protein by Western Blot method in pericontusional cerebral tissue samples. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. ***P < 0.001, compared to control group; + + +P < 0.01, significance value compared to trauma group.

Table 5
Western Blot values of Bax, Bcl-2 and Active Caspase-3.

	Control	Trauma	Trauma + EP
Bax/ß-Actin	0,46 ± 0,06	1,57 ± 013	0,88 ± 010
Bcl-2/ß-Actin	1,78 ± 004	0,38 ± 004	0,83 ± 0,07
Active Caspase-3/ß-Actin	0,36 ± 0,06	1,59 ± 010	0,96 ± 0,07





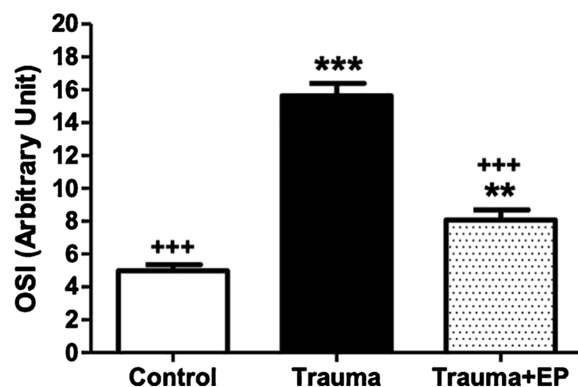


Fig. 21. Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) values. A: Control, B: Trauma, C: Trauma + Ethyl Pyruvate (EP) group. **P < 0.01, ***P < 0.001, compared to control group; + + +P < 0.001, significance value compared to trauma group.

Table 6
Mean ± standard deviation values of total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI).

	Control	Trauma	Trauma + EP
TAS (Trolox equivalent/L)	170 ± 0,12	0,88 ± 004	147 ± 010
TOS (μmol H_2O_2 Eqv./L)	808 ± 0,16	13,48 ± 0,23	11,31 ± 0,15
OSI (Arbitrary Unit)	497 ± 0,37	15,64 ± 0,75	808 ± 0,60

proinflammatory cytokine release from monocytes because of HMGB1 protein release in ischemic cerebral tissue. These reactive oxygen species induce protein and lipid oxidation (Andersson et al., 2000). In one study, an increase of inducible nitrite oxide synthase (iNOS) has been

shown in alveolar macrophages due to HMGB1 protein in acute lung injury (Ren et al., 2006). A limited number of publications have been found investigating the relationship between oxidative stress and HMGB1 protein in traumatic brain injury. Even though proinflammatory M1 microglia formed in the result of microglial activation increases the inflammation by increasing the release of cytokines and chemokines such as tumor necrosis factor (TNF), IL-6 and IL-1 beta, it increases oxidative stress by producing various neurotoxic mediators such as reactive nitrogen and oxygen radicals as a result of NADPH oxidase and iNOS expression. and, as a result of all these events, it is actively involved in the tissue destruction of the central nervous system (Rodriguez-Rodriguez et al., 2014; Goldmann and Prinz, 2013; Norden et al., 2015; Chen et al., 2014). The relationship between HMGB1 protein and oxidative stress was investigated in our study. It was found out that, when the total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) values in the pericontusional cerebral tissue samples, the TAS values were statistically significant low and the TOS and OSI values were significantly increased in the group where the HMGB1 protein level is increased. It was seen that the low HMGB1 protein levels in the group of ethyl pyruvate inhibition as well as the TAS values, which were previously low, are increased and the TOS and ISO values are decreased in the trauma group, and were become close to the control group values. As a result, post-traumatic HMGB1 protein was found to cause an increase in oxidative damage.

4.5. Ethyl pyruvate and HMGB1

Ethyl pyruvate (EP) is the first defined pharmacological inhibitor of HMGB1 secretion (Sims et al., 2001). Its protective efficacy against several diseases such as systemic inflammation (Ulloa et al., 2002) and stroke (Yu et al., 2005) has been shown. It has been found in many experimental disease models that ethyl pyruvate demonstrates its anti-inflammatory effect by inhibiting HMGB1 release and thus reduces cell damage (Yang et al., 2008; Shang et al., 2009). In a study that Wang et al established a model of spinal cord ischemia, they suggested that ethyl pyruvate prevents the release of HMGB1 and thus reduced the development of motor deficit and the number of apoptotic neurons (Wang et al., 2009). It has been found out that, ethyl pyruvate prolongs survival of rats with created systemic infection and lethal sepsis as well as reduces ischemia-induced myocardial damage (Ulloa et al., 2002; Woo et al., 2004). Ethyl pyruvate significantly reduced infarct volume in post-ischemic brain (Yu et al., 2005). It increased motor function scores in the models of traumatic brain injury and spinal cord ischemia (Su et al., 2011; Wang et al., 2009). In a study conducted on a model that established an experimental traumatic brain injury in rats by lowering weight, it has been seen that the neuroprotective effects of the ethyl pyruvate treatment are associated with inhibition of the HMGB1/TLR4/NF-kB pathway (Su et al., 2011). In a study, it has been seen that ethyl pyruvate has blocked the release of TNF, IL-6 and NO from active microglia and this inhibition has been considered responsible for the anti-inflammatory effect of ethyl pyruvate (Stanisavljević et al., 2015).

The effects of HMGB1 protein that plays a role in the inflammatory response in traumatic brain injury against other mechanisms such as cerebral edema, oxidative stress and apoptosis leading to secondary damage were specified, which were not indicated before in a same study. The effect of HMGB1 protein on tight linking molecules in the blood brain barrier and consequently cerebral edema in traumatic brain injury specified in this study has not been directly shown in other previous studies. In addition, the effects of HMGB1 protein on bax and bcl-2 shown in this study, which are apoptotic indicators, were not found in any previous study. We believe that these results will shed light on future studies.

4.6. Limitations

Our study has 2 basic limitations. Firstly, EP is used, which is well known with its effect and used mostly to inhibit HMGB1 protein. When searching the literature to look for the effects of HMGB1 protein, it is understood that the protective effects provided by ethyl pyruvate in the diseases mentioned above are in fact the effects of inhibition of HMGB1 protein. Considering that EP may have its own anti-inflammatory and antioxidant effects, other than inhibiting HMGB1 protein, future studies can be planned to compare EP with anti-HMGB1 antibody. Secondly, all rats were sacrificied at the 24th hour in which we think that HMGB1 showed maximal efficacy. Future studies can be planned, which compare the nuclear, time-based cytoplasmic and extracellular displacements and effects of HMGB1 protein, by performing sacrification processes in different hours.

5. Conclusion

In this study, it was found that HMGB1 protein that induces the inflammatory response by releasing from post-traumatic necrotic cells progresses cerebral edema by increasing the expressions of receptors TLR4 and RAGE and decreasing the occludin, claudin-5 and ZO-1 levels, which are the tight linking proteins in the blood-brain barrier, and influenced other post-traumatic secondary damage mechanisms in negative manner by increasing apoptosis and oxidative stress.

In conclusion, HMGB1 protein plays a key role in the pathogenesis of traumatic brain injury by inducing secondary damage mechanisms and it may be a potential target in the treatment of head trauma because of these characteristics.

Declaration of Competing Interest

The authors declare no competing financial interests and no sources of funding and support, including any for equipment and medications.

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