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Comparison of immunological, histological and oxidative effects of felbamate and levetiracetam in traumatic brain injury

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Abstract. – OBJECTIVE: We aimed to compare immunological, histological and oxidative effects of antiepileptic agents; felbamate and levetiracetam on head trauma in rats.

MATERIALS AND METHODS: In this study, 32 Sprague-Dawley genus male rats were used. A closed head trauma mechanism was constituted in order to perform head trauma in rats. Rats were divided into 4 groups, and each group had 8 rats. Following head trauma, Group 1 (Control); normal saline was administered, Group 2; levetiracetam 50 mg/kg was administered, Group 3; felbamate 100 mg/kg was administered, and Group 4; levetiracetam 50 mg/kg and felbamate 100 mg/kg were administered with a combination. Injections were administered intraperitoneally once a day for 20 days. The rats were decapitated at the end of the 20th day. Blood and tissue samples were collected and analyzed for biochemical, immunohistochemical and histological parameters.

RESULTS: Serum cytokine levels in Group 2, 3 and 4 were lower when compared to the control group. In Group 4, in which combined therapy was performed, cytokine levels were found to be the lowest. In Groups 2 and 3, a significant decrease in vascular congestion, mononuclear cell infiltration, hemorrhage, and neural degeneration was noticed in the pia mater. In Group 2, a decrease in vascular congestion and Purkinje cell degeneration was obtained in the cerebellum. However, the best outcomes were determined in Group 4.

CONCLUSIONS: We determined that levetiracetam and felbamate alone are useful with respect to immunological, oxidative and histological alterations. However, their utility is better when used in a combination.

Key Words:
Head trauma, Felbamate, Levetiracetam, Rat.

Introduction

Head trauma is the leading cause of death in patients with trauma in developed countries[1]. Even though head trauma is an important health issue with the economic burden, morbidity and mortality, studies in this field are limited[2,3].

Secondary brain damage occurs as a result of complex physiopathological events and arises within hours and days following primary brain damage. It was shown that the secondary brain damage is associated with a poor prognosis in patients with head trauma. Mechanisms involved in secondary brain damage are neurotransmitter release, gene activation, calcium-dependent cell damage, free radical formation, mitochondrial dysfunction and inflammation[4].

Lipid peroxidation (LP) plays an essential role in secondary brain damage. Prevention of free oxygen radical formation following ischemia and trauma in the Central Nervous System (CNS) decreases morbidity and mortality with their therapeutic effects[5].

Recurrent epileptic seizures secondary to brain damage following head trauma are defined as posttraumatic epilepsy. They may occur at any time following trauma[6]. As an antiepileptic, levetiracetam (Lev), was reported to be an effective

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alternative treatment choice in posttraumatic epilepsy with fewer side-effects[7]. It was also shown that Lev therapy is useful in molecular, histologic and behavioral aspects of neurological recovery after traumatic brain injury[8]. Felbamate (Fel), a new generation antiepileptic drug, has not been analyzed regarding its immunological, histological and oxidative effects after head trauma in the literature.

This study aims to compare the immunological, histological and oxidative effects of antiepileptic agents, Lev and Fel, following the head trauma in rats.

Materials and Methods

The Experimental Animal Research Laboratory at Inonu University provided 32 male Sprague-Dawley rats, 6-8 weeks old and weighing between 250-300 g, for this study. The study was conducted by the Ethical Standards and approved by our Institutional Animal Research Ethics Board (Acceptance No.: 2014/A-57). The animals were fed rat chow and water ad libitum. Before the trial, the rats were kept individually caged at room temperature with a 12 h light-dark cycle.

The head trauma method in which the skull was protected intact described by Marmarou et al[9] was performed to all rats. A trauma mechanism for this method was constituted by a pipe with a length of 2.15 m, an inner diameter of 19 mm, an outer diameter of 25 mm and a stabilizer attached to it. The rats were located in a place of 12×12×43 cm in dimensions with protective foam material, a metal disk of stainless steel of 10 mm in diameter, 3 mm in length, and 9 metallic segments of 18 mm in diameter and 50 g of weight. The hair on the head of rats was shaved. A scalp incision was performed in sterile conditions under local anesthesia. The frontoparietal region was exposed by a median vertical incision extending from the frontal region to the occipital region. Periost that covers vertex was dissected by a dissector. In order to make diffuse cranial damage by falling weights and have a broader affected area, a metal disc of stainless steel was fixated with dental acrylic in rats' vertexes between coronal and lambdoid sutures to fill the space between the disc and the vertex. Moderate head trauma was performed according to the Marmarou model by releasing a 450 gr weight from 1 m height onto the parietal regions of rats.

The rats were randomized into four study groups. Each group consisted of eight rats. After head trauma, no treatment was administered to the Group 1 (Control); Lev 50 mg/kg daily for 20 days was administered to the Group 2; Fel 100 mg/kg daily for 20 days was administered to the Group 3; Lev 50 mg/kg and Fel 100 mg/kg daily for 20 days were administered to Group 4. Injections were administered intraperitoneally. After the head trauma, all rats were resuscitated by subcutaneous administration of 10 ml/kg of an isotonic saline solution (0.9% NaCl). The rats were decapitated at the end of the 20th day.

The samples were stored at -20°C until thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) were analyzed from tissue specimens. Then, the samples were placed in glass tubes after weighing. In order to obtain a dilution of 1/10 (g/h), 1.15% potassium chloride was added and homogenized in a glass-teflon homogenizer for 3 minutes at 16000 rpm. Measurements of malondialdehyde (MDA) and protein levels were performed in the preparations. Remaining homogenates were centrifuged in 3500 rpm for 45 minutes at +4°C. The supernatants were used to measure GSH, GPx, and CAT enzymes. A mixture of chloroform/ethanol (3/5, w/w) was added in a proportion of 1/1 (w/w) to the remaining supernatant, and mixed by a vortex. Then, it was centrifuged at 3500 rpm for 45 minutes. In the chloroform/ethanol phase (upper phase), SOD enzyme activity and protein levels were measured.

Immunological studies were performed with the blood samples. Levels of serum cytokines, such as interleukin one beta (IL-1 beta), interleukin 4 (IL-4), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) were determined.

Tissue samples were fixed in 10% formaldehyde for histopathologic investigations. The routine tissue follow-up process was performed, and they were embedded in paraffin blocks. The blocks were cut into 5 μm thick sections, mounted on slides and stained with Hematoxylin-Eosin (H-E). Tissue samples were examined using a Leica DFC 280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

For statistical analyses, IBM SPSS Statistics 25.0 (IBM Corp., Armonk, NY, USA) for Windows was used. After Shapiro normality tests, one-way ANOVA was used for the comparison of groups. Multiple comparisons were carried out by

Table I. TNF- α, IL-4, IL-6 and IL-1 beta levels in rats (n=8, mean ± SE).

	IL-1 beta	IL-4	IL-6	Tnf-α
Group 1	600.8 ± 92.1[a]	301.1 ± 28.2[a]	21.8 ± 4.96[a]	256.2 ± 48.7[a]
Group 2	538.0 ± 58.3[a]	209.8 ± 25.5[b]	14.6 ± 4.64[b]	209.3 ± 33.5[ab]
Group 3	512.2 ± 95.9[a]	216.6 ± 35.1[b]	14.9 ± 4.80[b]	171.9 ± 47.0[b]
Group 4	369.3 ± 88.6[b]	197.8 ± 34.6[b]	11.5 ± 3.63[b]	163.0 ± 35.9[b]

Different letters in the same column present the statistical significance (p<0.01). Fel: Felbamate, Lev: Levetiracetam, IL-1 beta: Interleukin 1 beta, IL-4: Interleukin 4, IL-6: Interleukin 6, TNF-α: Tumour necrosis factor-alpha.

the Tukey test. Data was given mean ± standard error (SE) and p<0.05 values were considered as statistically significant.

Results

Immunological Analyses

All immunological findings were presented in Table I. In all groups, cytokine levels elevated after trauma. In Groups 2, 3 and 4, cytokine levels were lower than those of Group 1. In Group 4, in which combined therapy was performed, the lowest cytokine levels were obtained. The combined therapy in Group 4 was more effective than the therapies in Groups 2 and 3 (p<0.01).

While IL-1 beta levels did not decrease significantly in Groups 2 and 3, it decreased significantly in Group 4 (p<0.01). Between Groups 2 and 3, there was not a significant difference in respect to IL-1 beta levels. When compared to Group 1, IL-4 levels in Groups 2, 3 and 4 were significantly different (p<0.01). The highest decrease in IL-4 level was obtained in Group 4. When compared to Group 1, IL-6 levels in Groups 2, 3 and 4 were significantly different (p<0.01). The highest decrease in IL-6 level was obtained in Group 4. When compared to Group 1, TNF-α levels in Groups 3 and 4 were significantly different (p<0.01). The highest decrease in TNF-α level was obtained in Group 4.

Biochemical Analyses

The values for TBARS, SOD, CAT, GSH and GPx levels were given in Table II. In rats with head trauma, TBARS level as an indicator of oxidative damage was found to be higher in Group 1 when compared to the other groups (p<0.01). However, it was found that Lev and Fel treatment significantly inhibited the increase in TBARS caused by trauma, both individually and in combination (p<0.01). The decreases in TABRS levels in Groups 2 and 4 were found to be higher than Group 3.

A significant decrease in GSH, SOD, GPx, and CAT levels was determined in Group 1 compared to Group 2, 3 and 4 (p<0.01). Following drug treatment, SOD and GSH levels increased significantly in Group 2, 3 and 4 (p<0.01).

Histopathologic Analyses

In Group 1, there was significant histopathological damage in the brain tissue. There was vascular conjugation in the pia mater (Figure 1A); mononuclear cell infiltration (Figure 1B), hemorrhage (Figure 1C) and vascular conjugation in the cerebral cortex (Figure 1D) and a significant degeneration in the neurons of the cerebral cortex (Figure 1E).

In Group 2, there was a significant decrease in the vascular conjugation (Figure 2A), mono-

Table II. TBARS, SOD, CAT, GSH and GPx levels in rats (n=8, mean ± SE).

	ISOD U/mg protein	GSH nmol/ml	TBARS nmol/g	CAT k/mg protein	GPx U/mg protein
Group 1	31.0 ± 4.73[a]	198.0 ± 22.0[a]	9.76 ± 1.15[a]	0.026 ± 0.0011[a]	263.1 ± 10.3[a]
Group 2	54.2 ± 10.9[b]	235.3 ± 12.0[b]	6.76 ± 0.59[b]	0.018 ± 0.0012[b]	195.2 ± 12.7[b]
Group 3	46.9 ± 7.31[c]	247.3 ± 21.7[b]	7.72 ± 1.85[c]	0.019 ± 0.0014[b]	210.9 ± 15.9[c]
Group 4	66.9 ± 12.8b	230.3 ± 12.1b	6.03 ± 0.62[b]	0.015 ± 0.0012[c]	190.8 ± 13.1[b]

Different letters in the same column present the statistical significance (p<0.01). Lev: Levetiracetam, Fel: Felbamate, SOD: Superoxide dismutase, GSH: Reduced glutathione, TBARS: Thiobarbituric acid reactive substances, CAT: Catalase, GPx: Glutathione peroxidase.

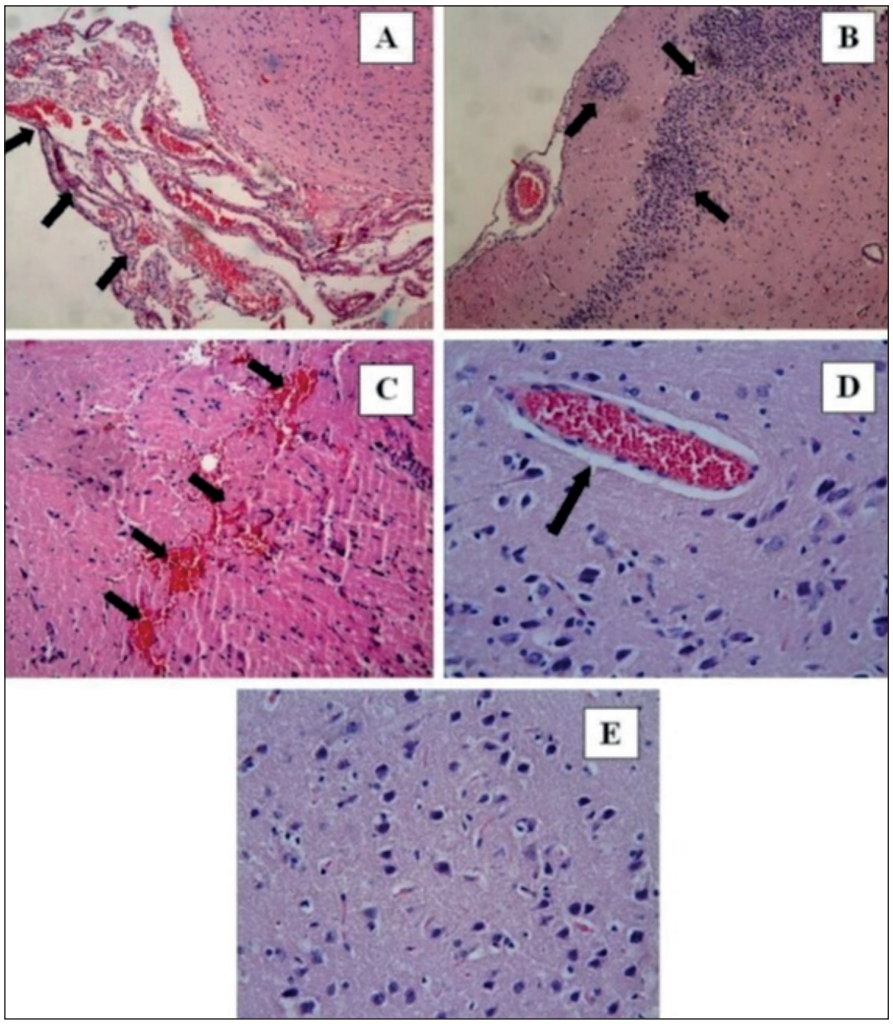

Figure 1. The histopathological images of Group 1 in the pia mater (**A, B,** ×10; **C,** ×20; **D, E,** ×40 magnification with H-E staining method).

nuclear cell infiltration (Figure 2B), hemorrhage and vascular conjugation (Figure 2C) and neuron degeneration in the pia mater (Figure 2D).

In Group 3, there was a significant decrease in vascular conjugation (Figure 3A), mononuclear cell infiltration (Figure 3B), hemorrhage (Figure 3C) and neuron degeneration (Figure 3D) in the pia mater.

In Group 4, the decreases in histopathological damage (Figure 4A, B) and neuron degeneration in the pia mater were more significant. Also, the neurons appeared normal (Figure 4B).

In Group 1, vascular conjugation in the cerebellum tissue (Figure 5A) and a significant degeneration in Purkinje cells (Figure 5B) were observed.

In Group 2 (Figure 6A, B) and Group 3 (Figure 6C, D), there were significant decreases in vascular congestion (Figure 6A, C) and Purkinje cell degeneration (Figure 6B, D) in the cerebellum tissue. In Group 4 (Figure 6E, F), the cerebellum tissue and also Purkinje cells (Figure 6F) were observed in the normal histological appearance.

Discussion

In this study, the neuroprotective effects of antiepileptic agents Lev and Fel were analyzed following the head trauma. The best outcomes were obtained with a combination of Lev and Fel. When Lev and Fel were administered in combination, a significant decrease in immunological markers (IL-1 beta, IL-4, IL-6 and TNF-α), less accumulation of TBARS and a significant increase in SOD and GSH levels were determined.

Histopathologically, the most significant decrease in vascular congestion, mononuclear cell

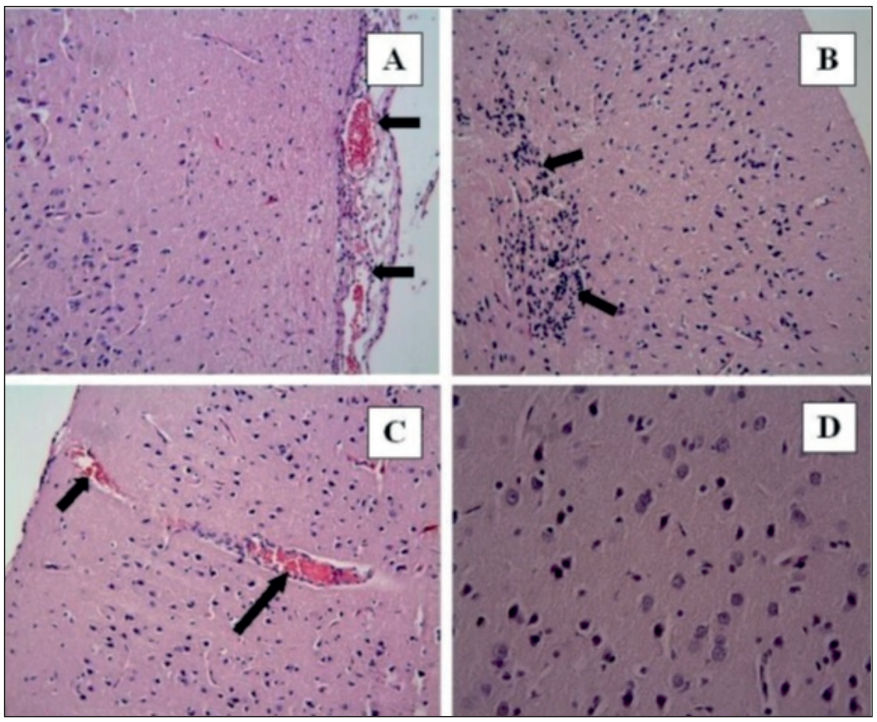

Figure 2. The histopathological images of Group 2 in the pia mater (**A, B, C,** ×20; **D,** ×40 magnification with H-E staining method).

infiltration, hemorrhage and neuron degeneration in pia mater was obtained in the group to which Lev and Fel were given in a combination. In this group, brain damage and neuron degeneration significantly decreased, and the neurons had a normal histological appearance. Additionally, the cerebellar tissue and Purkinje cells were in normal histological appearance.

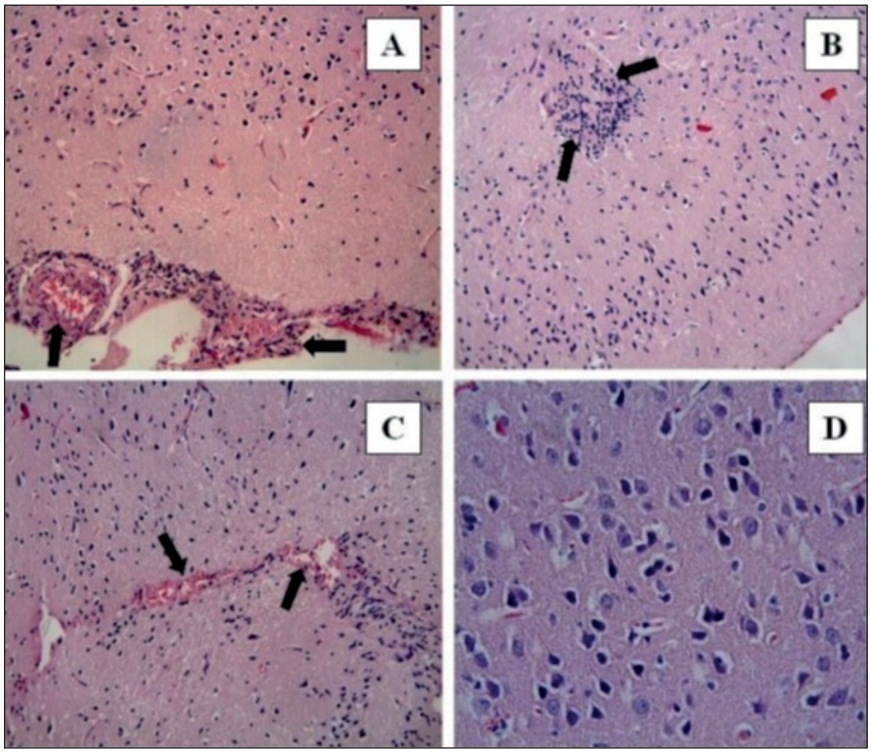

Figure 3. The histopathological images of Group 3 in the pia mater (**A, B, C,** ×20; **D,** ×40 magnification with H-E staining method).

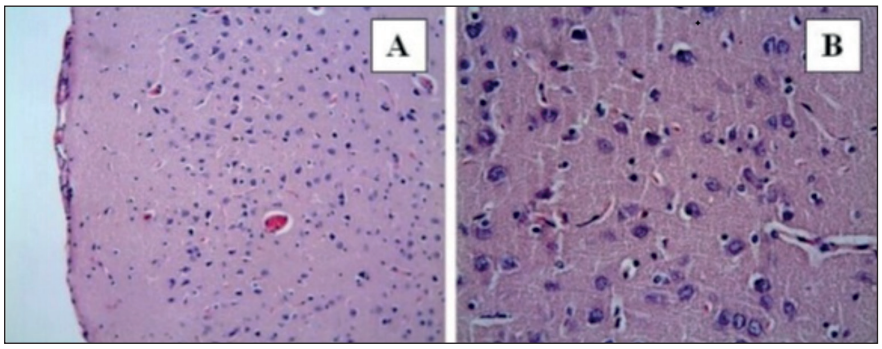

Figure 4. The histopathological images of Group 4 in the pia mater (**A,** ×20; **B,** ×40 magnification with H-E staining method).

Prophylaxis of posttraumatic epilepsy has great importance in preventing neurological sequelae. Lev was investigated as a prophylactic agent for epilepsy following traumatic brain injury. It was proposed as an alternative to phenytoin since it has usefulness in epilepsy following head trauma with fewer side effects[7]. Daily Lev treatment was shown to be useful on molecular, histological and behavioral fields of neurological recovery following traumatic brain injury[8]. In animal studies, besides controlling the seizures, Lev was shown to have neuron protective effects[10]. It was suggested that Lev increased the glutathione level in a dose-dependent manner, improved mitochondrial functions and thus, had neuron protective effects[11]. With Lev treatment, experimental brain damage performed by blocking the middle brain artery was decreased in adult rats[12]. Lev was also shown to decrease LP and oxidative stress in the hippocampus in seizures induced by pilocarpine in adult rats[13]. Marini et al[14] reported that Lev had a neuronal protective effect by reducing MDA levels.

Daily Lev treatment was investigated in neuroprotective, neuroplastic and neurobehavioral aspects and found to improve motor functions and had more protective effects on hippocampal cells in traumatic brain injury. Daily Lev use also decreased local IL-1 beta expression following traumatic brain injury. All these results show the beneficial effects of daily Lev treatment on histological, molecular and behavioral aspects of neurological recovery following a traumatic brain injury[8].

In our study, a decrease in IL-1 beta, IL-4, IL-6, and TNF-α levels was determined in the group to which Lev was given alone. Additionally, a significant decrease was determined in vascular congestion, mononuclear cell infiltration, hemorrhage and neuron degeneration in pia mater. In cerebellar tissue, vascular congestion and Purkinje cell degeneration were found to be decreased. The results represented in this work are consistent with the literature.

Neuron protective effects of Fel in the traumatic neuronal injury of the hippocampus were investigated and Fel treatment was found to

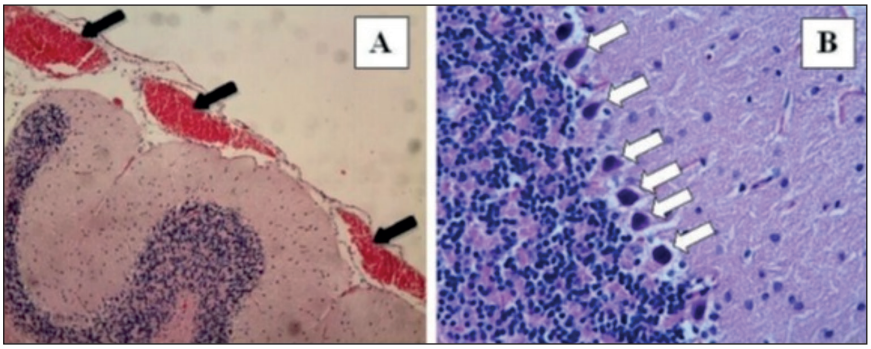

Figure 5. The histopathological images of Group 1 in the cerebellum tissue (**A,** ×10; **B,** ×40 magnification with H-E staining method).

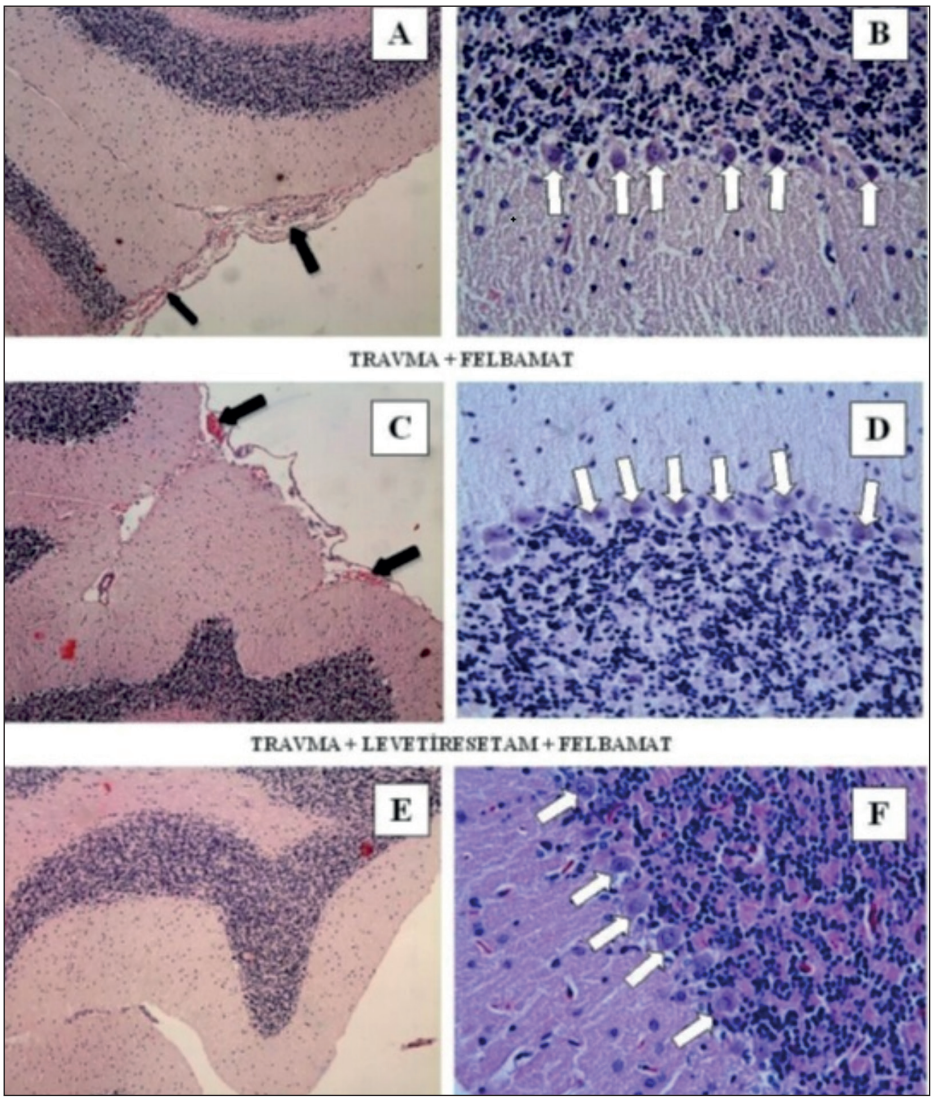

Figure 6. The histopathological images of Group 2, 3 and 4 in the cerebellum tissue (**A, C, E,** ×10; **B, D, F,** ×40 magnification with H-E staining method).

have a strong protective effect when compared to treatment without medication[15]. In our study, the levels of IL-1 beta, IL-4, IL-6 and TNF-α were found to be decreased in rats treated with Fel compared to the control group after head trauma. Besides, a marked decrease was determined in vascular congestion, mononuclear cell infiltration, hemorrhage, and neuronal degeneration in the pia mater layer of brain tissue. A similar decrease was also determined in vascular congestion and Purkinje cell degeneration in cerebellar tissue.

It was previously shown that TNF-α, IL-1, hydrogen ions, potassium ions, calcium ions, arachidonic acid and its metabolites, free oxygen radicals, histamine and quinine cause an increase in both vasogenic and cytotoxic brain edema in damaged tissue following traumatic brain injury[16]. In the experimental diffuse brain damage model, it was shown that the proinflammatory cytokine IL-1 beta infiltrated the damage zone and its serum levels increased in brain tissue within 24 hours[17]. TNF-α was shown to play an essential role in traumatic brain damage and cause glial, microglial, astrocytic and neuronal damage[18,19].

It was reported that hydroxyl (OH) radical increases just after trauma, cause damage to vascular endothelium, and initiate the LP in the brain membrane by damaging blood-brain barrier[20]. It was shown that the levels of MDA as a catabolite and an important indicator of LP, increase in the acute phase after experimental closed head trauma[21]. In a study on head injury, MDA level was higher in trauma performed control group compared to the non-traumatic sham group[22].

In our study, TBARS levels as an indicator of oxidative damage were shown to increase significantly in the control group when compared to the other groups in traumatized rats. It was determined that Lev and Fel treatment both together and alone decreased trauma-related TBARS levels significantly. The highest decrease was obtained in the group to which Lev and Fel were administered together. In treatment groups, GSH and SOD levels were elevated significantly. These results have shown that, together or alone, both agents prevented secondary damage in head trauma; however, the usefulness of Lev and Fel administration together had the highest rate of the antioxidant effect.

In a study, in traumatized brain parenchyma tissue; edema, vascular congestion, neuronal injury indicators, such as hyperchromasia in neuronal nuclei, nuclear pyknosis, eosinophilic degeneration in the cytoplasm, axonal edema, and focal neuronal loss and gliosis in trauma area were determined. In the same study, coenzyme Q 10, a powerful antioxidant that plays a role in membrane stabilization, caused significantly less neuronal degeneration[23].

In our study, vascular congestion, mononuclear cell infiltration, hemorrhage and marked degeneration in neurons of the cerebral cortex were observed in the pia mater layer of the brain tissue of rats in the control group. In the control group, also vascular congestion and marked Purkinje cell degeneration were detected in cerebellum tissue. In Lev or Fel administered groups, a distinct decrease in vascular congestion, mononuclear cell infiltration, hemorrhage, and neuron degeneration in pia mater layer of brain tissue was noticed. In these groups, a decrease in vascular congestion and Purkinje cell degeneration was observed in cerebellar tissue. In the group that Lev and Fel were administered together, a very evident decrease in neuron degeneration in brain tissue and normal histological appearance of neurons were determined. Additionally, the normal histological appearance of Purkinje cells and cerebellum tissue was detected.

The information about the effects of Fel, known as an antiepileptic drug, on head trauma is quite insufficient in the literature. In this study, we investigated Lev and Fel, used alone or in a combination, with respect to their protective effects in head trauma. Our study supports the use of the drugs for their protective effects in clinical practice following head trauma. Therefore, we think that it is a valuable study with novelties. However, further investigations are needed for the clinical use of these medications.

Conclusions

Our study revealed that Lev and Fel improved histological, immunological and biochemical parameters when administered alone; however, the best results were obtained when Lev and Fel were administered together following head trauma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Statement of Institutional Review Board Approval

The study was conducted by Ethical Standards and approved by Our Institutional Animal Research Ethics Board (Acceptance No. 2014/A-57).

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