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Research Article

Therapeutic effects of Lacosamide in a rat model of traumatic brain injury: A histological, biochemical and electroencephalography monitoring study ☆

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ABSTRACT

Objective: Traumatic Brain Injury (TBI) is a major cause of death and disability worldwide, especially in children and young adults. TBI can be classified based on severity, mechanism or other features. Inflammation, apoptosis, oxidative stress, and ischemia are some of the important pathophys-iological mechanisms underlying neuronal loss after TBI. Lacosamide (LCM) is an anticonvulsant compound approved for the adjunctive treatment of partial-onset seizures and neuropathic pain. This study aimed to investigate possible neuroprotective effects of LCM in a rat model of TBI.

Material and methods: Twenty-eight adult male, Wistar albino rats were used. The rats were divided into 4 groups. Group 1 was the control group (n=7). Group 2 was the trauma group (n=7) where rats were treated with 100 mg/kg saline intraperitoneally (IP) twice a day. Groups 3 and 4, rats were treated with 6 (group 3, n=7) or 20 (group 4, n=7) mg/kg Lacosamide IP twice a day. For each group, brain samples were collected 72 hours after injury. Brain samples and blood were evaluated with histopathological and biochemical methods. In addition, electroencephalograpy monitoring results were compared.

Results: The immunoreactivity of both iNOS and eNOS (oxidative stress markers) were decreased with LCM treatment compared to trauma group. The results were statistically significant (***P<0.001). The treatments of low (56,17±9,69) and high-dose LCM (43,91±9,09) were decreased the distribution of HIF-1α compared to trauma group (P<0.01). The number of apoptotic cells were decreased with LCM treatment the difference between the trauma group and 20mg/kg LCM treated group (9,55±1,02) was statistically significant (***P<0.001). Malondialdehyde level was reduced with LCM treatment. MDA level was significantly higher in trauma group compared to LCM treated groups (***P<0.001). The level of Superoxide dismutase in the trauma group was 1,86 U/ml, whereas it was 36,85 U/ml in 20mg/kg LCM treated group (***P<0.001). Delta strength of EEG in 20mg/kg LCM treated group were similar to control group values after LCM treatment.

Conclusion: No existing study has produced results suggesting that different doses of LCM has therapeutic effect against TBI, using EEG recording in addition to histological and biochemical evaluations in rats.

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Introduction

A traumatic brain injury (TBI) is defined by the Centers for Disease Control and Prevention as an alteration in the normal brain functions caused by blunt or penetrating trauma [1]. Traumatic brain injury is a major global and domestic health issue affecting more than 2.5 million people in the United States every year, with more than 5 million Americans currently living with at least 1 TBI related sequela [2]. While increasing understanding of the clinical characteristics and the underlying complex pathophysiological mechanisms of TBI has led to the development of novel and promising therapeutic approaches that show promising effects in preclinical studies and phase I/II trials, majority of them were unsuccessful in phase III clinical trials. In fact, more than 30 clinical trials of TBI pharmaceutical agents for diagnostics or therapeutic purposes have failed over the past three decades [3].

Here, we investigated Lacosamide (LCM) for a clinically translatable acute pharmacologic intervention for TBI. LCM is a strong newer antiepileptic drug which has a novel dual mechanism of action, that is, selective enhancement of sodium channel slow inactivation and modulation of collapsin response mediator protein-2 (CRMP-2) activity. Preclinical and clinical studies have demonstrated that LCM protects against seizures [4,5,6]. Furthermore, its anti-hyperalgesic and anti-apoptotic effects have been demonstrated with *in vitro* and *in vivo* studies [4,7]. Recently, limited studies have shown that, LCM attenuated brain damage in ischemic and traumatic animal models [8,9,10,11]. The present study was designed to investigate whether LCM treatment has the therapeutic effects in a rat model of TBI using histological and biochemical methods as well as electroencephalography (EEG) monitoring.

Material and methods

Experimental Protocol

In the present study, 28 adult, male, Wistar albino rats (200 ± 50g) were used. The rats were kept under a temperature of 22°C, humidity of 65%, and light–dark cycles of 12:12 h, and they had *ad libitum* access to laboratory standard food and water. This study was approved by The Animal Experiments Local Ethics Committee of Manisa Celal Bayar University (06/12/2016/77.637.435-76), Turkey. The rats were grouped as follows: Group 1, control group (n=7), skin incision only: a skin incision was made, and non-traumatic brain samples were collected 72 hours after surgery. Group 2, trauma group (n=7): TBI was induced as described below, and rats were treated with 100 µL saline intraperitoneally (IP) twice a day. After craniectomy, brain samples were collected 72 hours after injury. Groups 3 and 4: TBI was induced, rats were treated with 6 (Group 3, n=7) or 20 (Group 4, n=7) mg/kg LCM IP twice a day (LCM was injected in 100 µL saline), and brain samples were collected 72 hours after injury. The rats in the treated groups (Groups 2, 3 and 4) received the first dose immediately after trauma and other doses were given at 12-hour intervals for 72 hours. For all treated groups vehicle or LCM were given with a total of 6 doses.

Anesthesia and Trauma Procedure:

Before the surgical procedure, the rats received an IP injection of 10 mg/kg xylazine (Bayer Birleşik Alman Ilaç Fabrikaları, Istanbul, Turkey) and 75 mg/kg ketamine hydrochloride (Parke Davis, Istanbul, Turkey). TBI was induced using the falling weight technique described by Marmarou et al. and modified by Ucar et al. [12]. Briefly, each anesthetized rat's scalp was shaved, a midline incision was made, and the periosteum was retracted. A 2-mm thick metallic disc was used as the helmet. The steel disc was fixed to the skull's central portion using bone wax. The rats were placed in the prone position on a foam bed. An inflexible rope was tied to the weight to prevent repeated impacts. A 350-g steel weight was dropped through a 1-m vertical section of the plexiglass tube. The electroencephalography (EEG) monitoring was performed before and after LCM administrations of 1^{st}, 2^{nd}, 4^{th} and 6^{th} doses. And after 3 days, the rats were sacrificed. The brain tissues were used for histochemical, immunohistochemical, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays [13], and intracardiac blood samples were collected for the biochemical anaysis of superoxide dismutase (SOD) and malondialdehyde (MDA) [14].

Electroencephalography (EEG) monitoring

After head trauma, EEG monitoring was performed before and after LCM administrations of 1^{st}, 2^{nd}, 4^{th} and 6^{th} doses. For this purpose, the rats were fixed on the stereotaxy device. A rostrocaudal incision was made in the scalp. Totaly 3 screw electrodes were placed to scalp. Briefly, two screw electrodes were placed in the right somatocortex skull bone. The first screw electrode was placed to 3mm lateral and 4mm rostral to bregma and the second screw electrode was placed to 3mm lateral and 4mm caudal to bregma. Finally, reference electrode was placed to 3 mm lateral and 4 mm caudal to bregma on left-somatocortex skull bone [15,16]. Then the electrodes were fixed to the skull with dental acrylic to prevent open area. Records were taken and analyzed using Powerlab / SP8 ADInstruments (Australia) recording system as 10,000 samples per second. Fourier analysis was applied by quantitative EEG method for digitalization of recorded EEG waves. (Digitization of observational data was provided.) Wave ranges were determined as: Delta: 0.5-4 Hz, Theta: 4-8 Hz, Alpha: 8-12 Hz and Beta 12-30 Hz.

Biochemical Evaluations

Before sacrificing the animals, cardiac blood samples were centrifuged for 5 minutes at 4,500 r/min at 4°C to separate the serum and plasma. To detect the superoxide dismutase (SOD) enzyme, the principle of this method is based on the reduction of nitroblue tetrazolium (NBT) by the superoxide producer xanthine - xanthokinase system. SOD activity was expressed as unit / g (U / ml) tissue protein. Lipid peroxidation measurement was carried out using the Esterbauer method. Malondialdehyde (MDA), which reacts with thiobarbutyric acid at 90 - 95 ° C, forms a pink chromogen. After fifteen minutes, the samples were cooled rapidly and their absorbances were read spectrophotometrically at 532 nm. The values obtained were expressed in µmol / g protein [14].

Histochemistry

The brain tissue sections were fixed in 10% formalin solution for 24–48 h. The routin parafine embedding procedure was applied to the tissue samples and they were embedded in parafine blocks. Then 5 µm thickness sections were cut using a microtome (RM 2135, Leica), and the histochemical, immunohistochemical, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed.

For the histochemical examination, tissue sections were dyed with routin haematoxylin and eosin (HE) procedure after the dewaxing and dehyration stages. The slides were mounted with entellan (UN 1866; Merck, Darmstadt, Germany). The histopathologi-

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cal evaluation was made by a blinded observer using an Olympus BX40 light microscope, and the images of tissue sections were photographed (SC50, Olympus, Germany) [13].

Immunohistochemistry

The brain tissue sections were deparaffinized at 60°C overnight and then in xylene for 30 min. Following the dehyration in ethanol, samples were then washed in phosphate buffered saline (PBS) and were treated with 0.5% trypsin solution (800.729.8350, ScyTek Laboratories, Inc., Logan, UT, USA) for 15 min at 37°C. The endogenous peroxidase activity was inhibited using 3% hydrogen peroxide. Following the washing in PBS, blocking solution (TA-125-UB; Lab Vision, Fremont, CA, USA) was applied to the samples for 1 h, and the sections were incubated with the primary antibodies anti-endothelial nitric oxide synthase (anti-eNOS; sc-654, Santa Cruz Biotechnology), anti-inducible nitric oxide synthase (anti-iNOS; GTX15322, GeneTex), anti-vascular endothelial growth factor (anti-VEGF; ab1316, Abcam), anti-neuronal nuclei (Neu-N, ab104225, Abcam) and anti-hypoxia-inducible factor 1-alpha (anti-HIF-1α, ab2185, Abcam) for 18 hours at 4°C. For negative control, the samples were not treated with the primary antibodies. After washing in PBS, the anti-mouse biotin-streptavidin hydrogen peroxidase secondary antibody (85-9043 Zymed Histostain kit; Zymed, San Francisco, CA, USA) was applied to the sections for 30 min, and sections were washed again in PBS. The diaminobenzidine (DAB; ScyTek) staining was performed to determine the appearance of the immunohistochemical reaction. For the counterstaining, Mayer's hematoxylin (72804E; Microm, Walldorf, Germany) was used, and the sections were mounted with entellan. The differences between the groups were stated by three independent researchers under an Olympus BX40 (Tokyo, Japan) light microscope [13]. The immunohistochemical procedure was performed three times, and the immunohistochemical data was given as H-score. The evaluation of stained samples were done as; weak (+), moderate (++) and strong (+++) respectively, and immunopositive cells were counted for each staining degree. The H-score formula was used: H-Score = Pi (intensity of staining + 1). Pi means the percentage of stained cells for each intensity (from 0% to 100%) [17].

TUNEL Assay

For the detection of apoptosis, an ApopTag Plus Peroxidase in situ apoptosis detection kit (S7101; Millipore, Billerica, MA, USA) was used according to the manufacturer's instructions. The brain tissue samples were treated with 20 µg/mL proteinase K treatment for 10 min at 37°C, and they were washed in PBS. After the treatment with an equilibration buffer, samples were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme for 1 h, at 37°C. Following the washing in Stop Wash Buffer for 10 min, an anti-digoxigenin peroxidase conjugate was applied to the samples for 30 min. Then the apoptotic cells were visualized using the diaminobenzidine (DAB; ScyTek), and the counterstaining was performed with Mayer's hematoxylin (72804E; Microm, Walldorf, Germany). The apoptotic cells were counted in five choosen areas, and **apoptotic** index was calculated using the formula: (number of apoptotic cells / total number of cells) × 100 [18].

Statistical Analysis

The results of all assays were analyzed on GraphPad (GraphPad Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA). The data were given as mean ± standard deviation. The $p \leq 0.05$ value was considered significant statistically [13].

Results

Histopathological Findings

Posttraumatic changes were investigated by comparing Dentate Gyrus, Subiculum and Hippocampus areas after hemotoxylin and eosin staining. In the control group, several rows of densely arranged cells, small pyramidal, glial cells in the molecular layer were observed. In the trauma group; various degenerative changes such as decrease in granular cell density and layer thickness, disorganization in small pyramidal cells, increased vacuolization in granular and molecular layer, pycnosis in pyramidal cells and shrinkage and deformities in neurons were observed. However in LCM treated groups; the degenerative changes were significantly reduced. Particularly, findings in high-dose (20 mg/kg) LCM treated group were similar to control group. Namely,in low-dose (6 mg/kg) LCM treated group; compacting in granular cell layer, pycnosis in granular, molecular and polymorphic layers were observed. In high-dose LCM treated group; increase in granular cell layer thickness and decrease in vacuolization were observed. In addition, the structure and organization of the capillaries in high-dose LCM treated group were close to control group **(Fig-1).**

Immunohistochemical Findings

The immunohistochemistry assay was performed to determine oxidative stress, hypoxia, vascularization and neuronal loss via eNOS and iNOS, HIF-1α, VEGF and Neu-N respectively, **and the results of H-score were given in Fig-5A.**

Oxidative stress is known to play a role in the secondary injury mechanism following TBI. In this study, we evaluated the oxidative stress via using iNOS and eNOS markers. The distribution of iNOS was increased in trauma group (81,04±6,11) compared to control group (40,11±4,43) (***P<0.001). Also, the differences between the control group and the groups treated with 6 mg/kg LCM (63,34±7,42) (***P<0.001) and 20 mg/kg LCM (55,04±4,77) were statistically significant (***P<0.001). In the comparision of trauma and LCM treated groups, the immunoreactivity of iNOS was diminished by LCM treatment and it was statistically significant (***P<0.001). Besides, there was no statistically significant difference between the LCM treated groups (p>0.05) (**Fig-2A**). The immunoreactivity of eNOS was enhanced in the trauma group (78,58±8,17) compared to control group (43,83±7,85) (***P<0.001). It was seen that LCM decreased the distribution of eNOS in trauma group compared to the treatment groups (***P<0.001). The H-score values were determined as 62,91±8,48 for the low-dose LCM group, and 53,83±7,65 for the high-dose LCM group. And there were significant differences between the control group and the low-dose LCM group (***P<0.001) and the high-dose LCM group (*P<0.05) (Fig-2B). Both iNOS and eNOS positive cells were lowest in the control group and highest in the trauma group. LCM treatment decreased the number of iNOS and eNOS positive cells with dose-dependent manner (**Fig-5A**).

The hypoxia was found to be increased by TBI in the trauma group (70,08±9,81) compared to control group (36,58±6,98) using HIF-1α marker (***P<0.001). The treatments of low (56,17±9,69) and high-dose LCM (43,91±9,09) were decreased the distribution of HIF-1α compared to trauma group (**P<0.01). The immunoreactivity levels of HIF-1α were similar in the control group and high-dose LCM group (P>0.05), whereas there was a significant difference between the control group and low-dose LCM group (**P<0.01) (**Fig-3A**). According to these findings, high-dose LCM treatment played a more effective role in reducing hypoxia (**Fig-5A**).

When the samples from all groups were examined in terms of vascularization, the VEGF immunoreactivity was the highest in

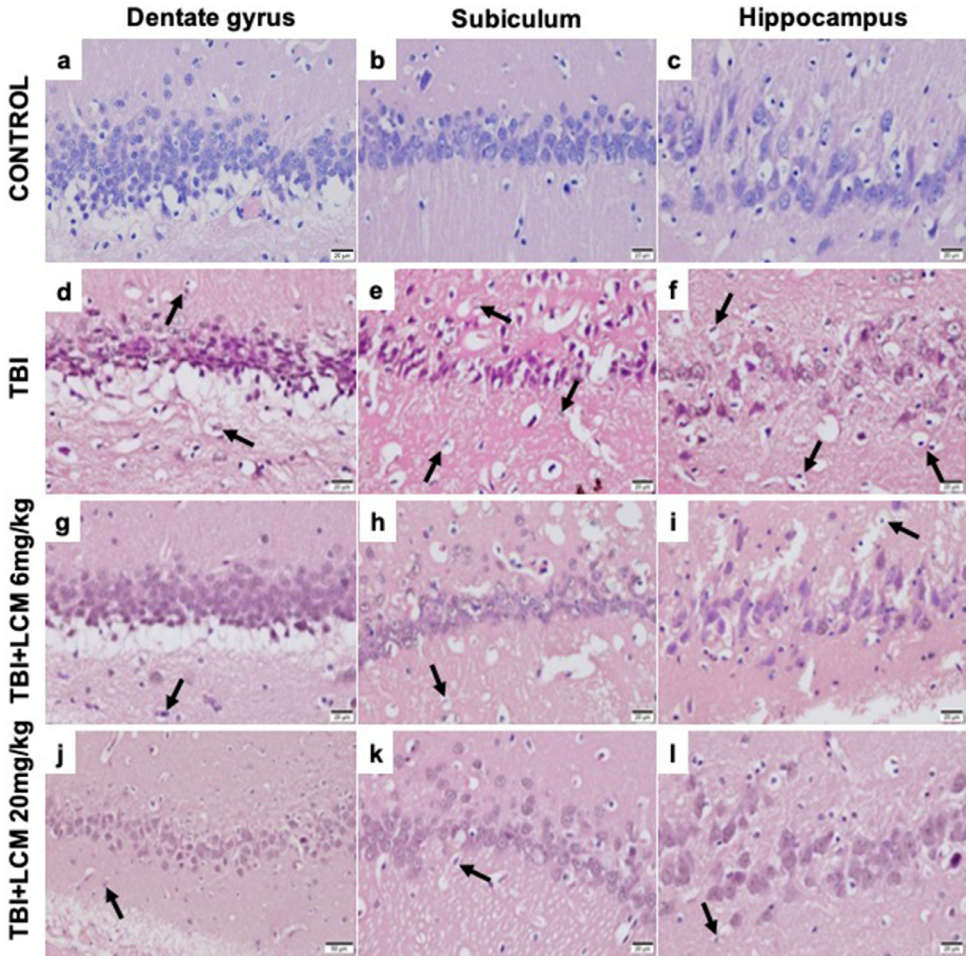


Fig. 1. Hematoxylin & eosin (H&E) staining for each group. Decrease in granular cell density and layer thickness, disorganization in small pyramidal cells, picnosis in pyramidal cells and shrinkage and deformities in neurons were observed in TBI compared to the control group **(a-f)**. In LCM treated samples **(g-l)** traumatic changes were less intense than in the trauma group **(d-f).** Scale bars: 20 μm.

the control group (126,6±11,17) and lowest in the trauma group (55,08±8,04) (***P<0.001). The increases of VEGF were statistically significant in the low-dose LCM group (88,33±10,45) (*P<0.05), and the high-dose LCM group (94,56±10,88) (*P<0.05) compared to trauma group (**Fig-3B**). On the other hand, the difference between the low and high-dose LCM groups was not significant (P<0.05).

For Neu-N which used in the immunohistochemical research of neuronal differentiation to assess the functional state of neurons, TBI caused a decrease in the immunoreactivity of Neu-N in the trauma group (85,67±8,50) compared to control group (135,66±12,00) (***P<0.001) (**Fig-4A**). Neu-N increase with high-dose LCM treatment. Namely, there was significant difference between the trauma group and the high-dose LCM group (108,51±10,80) (***P<0.001), whereas the difference between the trauma group and the low-dose LCM group (95,66±9,05) was not significant (P>0.05) (**Fig-5A**).

Apoptosis

The apoptosis was evaluated in all groups, and apoptotic index was given in Fig-5B. It was stated that; the number of apoptotic cells were higher in the trauma group (11,25±1,05) compared to control group and this was statistically significant (7,15±1,12) (***P<0.001). However, the difference between trauma group and the low-dose LCM group (10,10±1,50) was not significant (P>0.05). Similarly, there was no significant difference between low and high-dose LCM groups (P>0.05), but the difference between the trauma group and high-dose LCM group (9,55±1,02) was statistically significant (***P<0.001). Apoptotic index was the highest in the trauma group and decreased with LCM treatment in a dose-dependent manner (Fig-4B). These results might show that LCM treatment has a preventive effect against cell death in the neuronal tissue after TBI.

Table 1
Serum SOD and MDA levels (mean ± SD) for each group are seen.

GROUPS	SOD (U/ml)	MDA (µmol/L)
CONTROL	10,37 ± 1,86	3,58 ± 2,44
TBI	1,86 ± 0,85	47,59 ± 1,67
TBI+LCM 6mg/kg	20,14 ± 2,86	32,45 ± 7,72
TBI+LCM 20 mg/kg	36,85 ± 10,77	15,06 ± 1,28

Biochemical Findings

The mean serum level of MDA (as an oxidative stress marker) in the trauma group was **47,59 ± 1,67** μmol/L. The MDA level was reduced in the low (**32,45 ± 7,72** μmol/L) and high-dose (**15,06 ± 1,28** μmol/L) LCM groups, in a dose-dependent manner. MDA level was significantly higher in trauma group compared to low and high-dose LCM groups (***P<0.001) (Table 1). The level of anti-oxidant enzyme, SOD, in the trauma group was **1,86 ± 0,85**

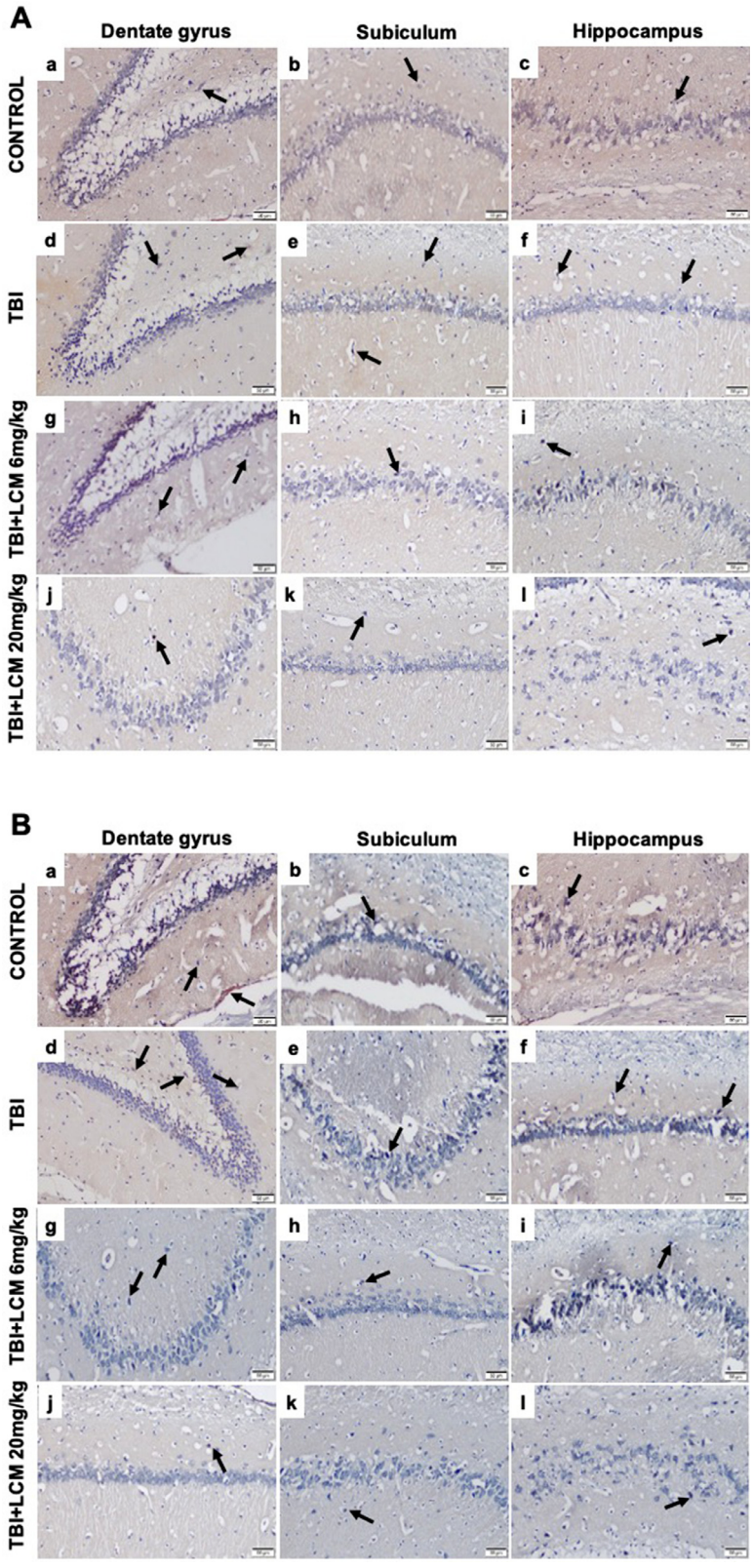


Fig. 2. Immunohistochemical staining of iNOS positive **(A)** and eNOS positive **(B)** cells are seen. iNOS and eNOS positive cells were highest in TBI group and decreased with LCM treatment. Scale bars: 50 μm

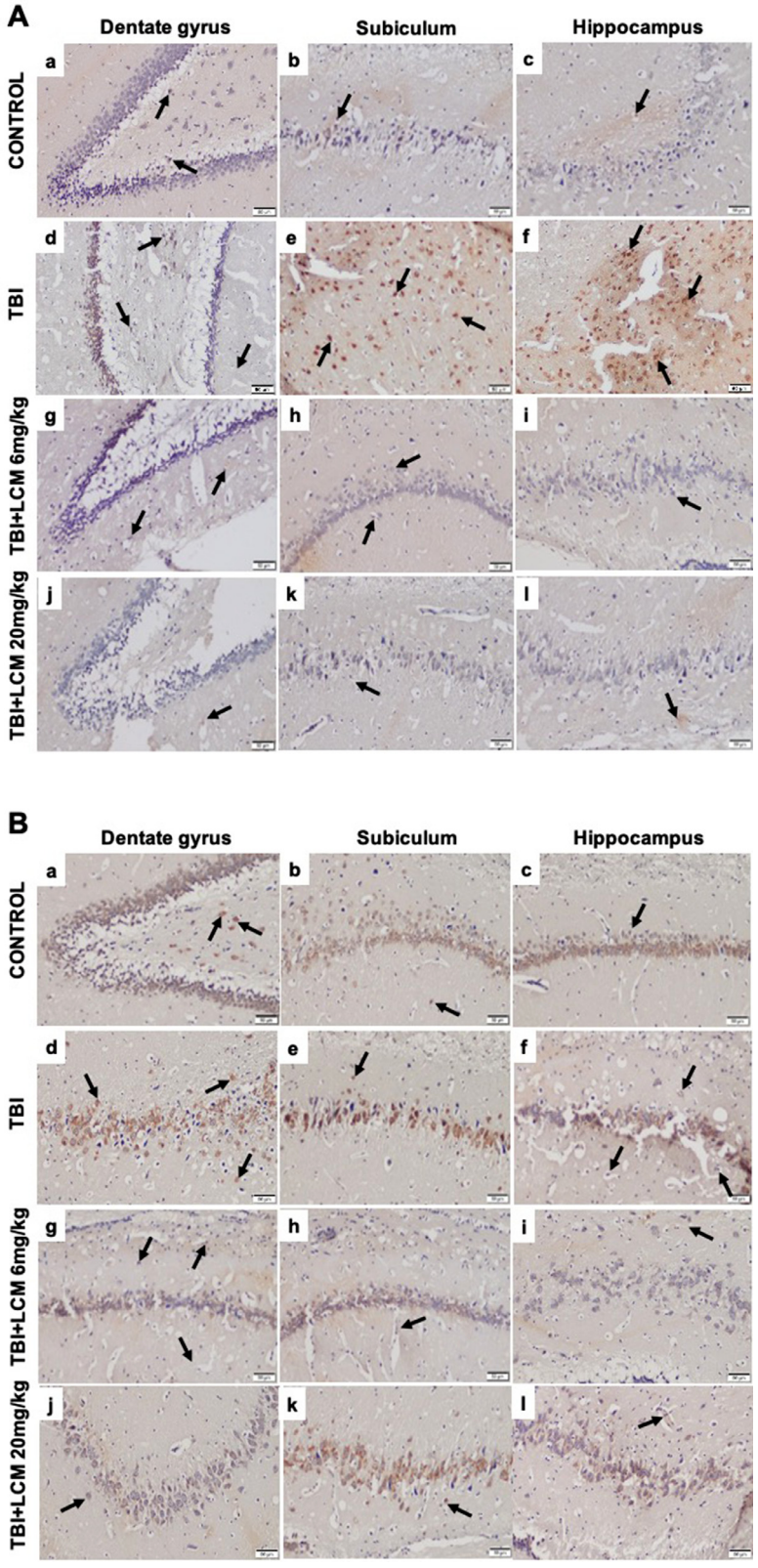


Fig. 3. Immunohistochemical staining of HIF-1α positive **(A)** and VEGF positive **(B)** cells are seen. HIF-1α positive cells were decreased after LCM treatment with dose dependent manner **(A)**. VEGF positive cells were decreased after TBI and increased after LCM treatment with dose dependent manner **(B)**. Scale bars: 50 μm

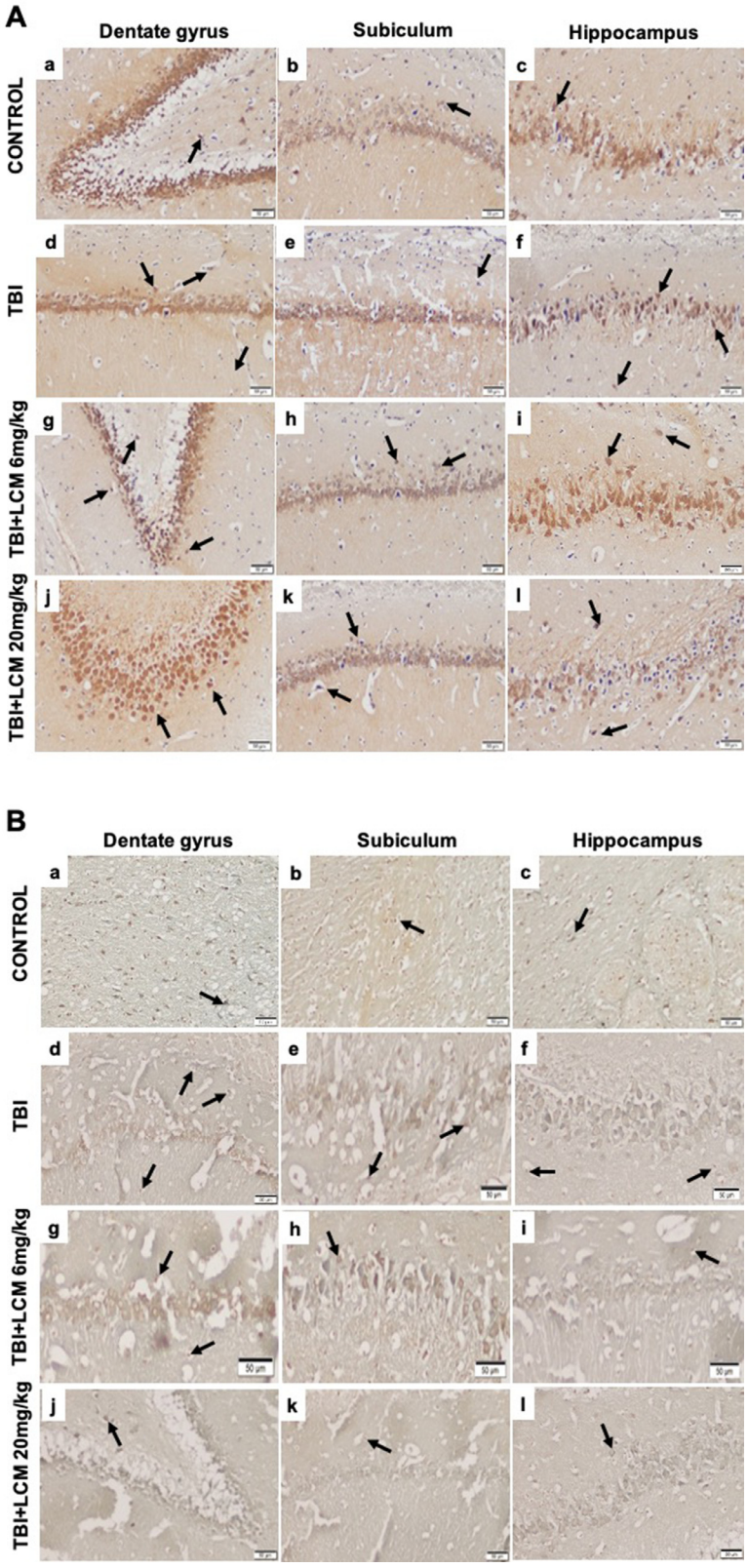
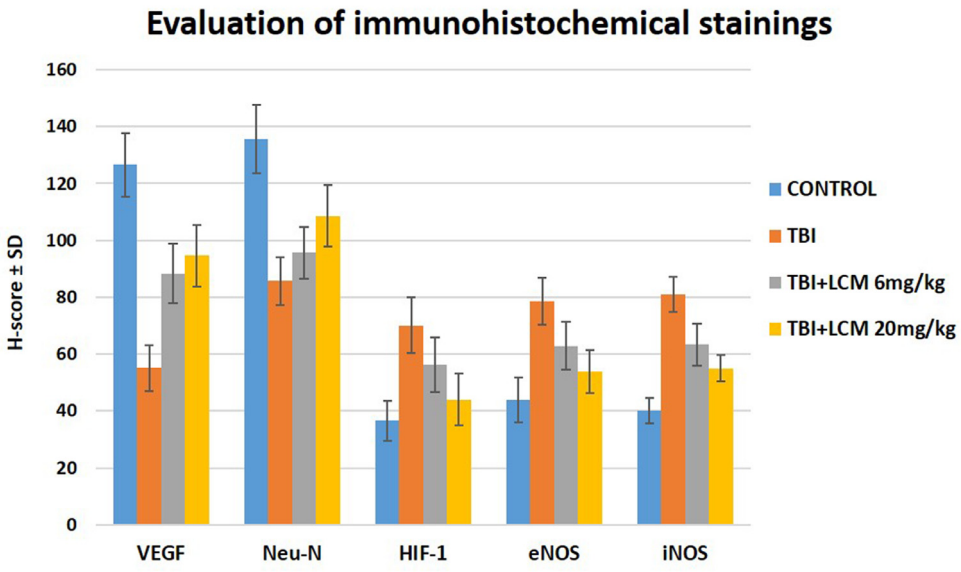


Fig. 4. Immunohistochemical staining of Neu-N positive **(A)** and TUNEL positive **(B)** positive cells are seen. Neu-N positive cells were increased after LCM treatment with dose dependent manner **(A)**. TUNEL positive cells for each group are seen. Apoptotic cells with pyknotic nuclei and apoptotic bodies were decreased after LCM treatment **(B).** Scale bars: 50 μm

A



B

GROUPS	APOPTOTIC INDEX
CONTROL	7,15 ± 1,12
TBI	11,25 ± 1,05
TBI+LCM 6mg/kg	10,10 ± 1,50
TBI+LCM 20mg/kg	9,55 ± 1,02

Fig. 5. Comparison of immunohistochemical staining **(A)** and apoptotic index **(B)** for each group is seen. Vascularization and neurons were increased in LCM treated groups compared to TBI group. Oxidative stress and hypoxia were decreased in LCM treated groups compared to TBI group **(A)**. The significant decrease in apoptotic index after LCM treatment with dose dependent manner compared to TBI group is seen **(B)**.

U/ml, whereas it was **36,85 ± 10,77** U/ml in the high-dose LCM group (***P<0.001). Also, the level of SOD was enhanced with the low-dose LCM (**20,14 ± 2,86** U/ml) treatment (**Table 1**). It was revealed that LCM has an anti-oxidant effect by increasing the SOD level.

EEG Recording Findings

The method of EEG measurement from rats was showed in Fig-6A and B. EEG changes were evaluated in terms of "spectral power density" (SPD) parameters of EEG frequency bands (Fig-6). Trauma and drug dose-related changings were more dramatic in the low frequency bands, particularly in the delta band (0.5- 4 Hz). **Fig. 6C** shows the effect of 6 mg/kg and 20 mg/kg LCM treatment on EEG delta band power spectral density. After trauma, increased delta strength decreased due to repeated doses of 6 mg/kg and 20mg/kg LCM treatment. Delta strength returned to control group values when given the 4th and 1st dose of 6 mg/kg and 20 mg/kg LCM treatment respectively (**Fig-6D**).

Discussion

In this study, we investigated the neuroprotective effect of LCM on TBI model in rats by histopathologic, immunohistochemical, biochemical and EEG monitoring assays. The parameters such as oxidative stress, hypoxia, vascularization, neuronal loss and apoptosis were used. We demonstrated that LCM has a recovery effect on these parameters. Especially, it has been found that it prevented the neuronal cell loss and supported vascularization by reducing oxidative stress, which has been increased with TBI. Together, the increase of SOD and the decrease of MDA were in parallel with the other findings. Therewithal, EEG monitoring assay showed that the brain activity reached a value close to the control group after LCM (6 mg/kg and 20mg/kg) treatment in rats in TBI model.

TBI has two distinct phases: primary and secondary injury. Primary injury occurs at the time of head impact and causes direct damage to neural tissue. Focal intracranial hemorrhage, extra-axial hematomas, brain contusion, and and diffuses axonal injury are all examples of primary lesions. The secondary injury can happen from minutes to days from the primary impact and consists of a molecular, chemical, and inflammatory cascade responsible for further cerebral damage [2,19,20]. At the cellular level, secondary injury is mediated by several pathways including, but not limited to; (a) excitotoxicity caused by an excess of the neurotransmitter glutamate and aspartate, (b) free radicals that results in degradation of proteins and phospholipid membranes of neural cells and (c) the neuroinflammatory response comprised of local and systemic immune activation [2,19,20]. The brain's inflammatory response to trauma is multifactorial and consists of the

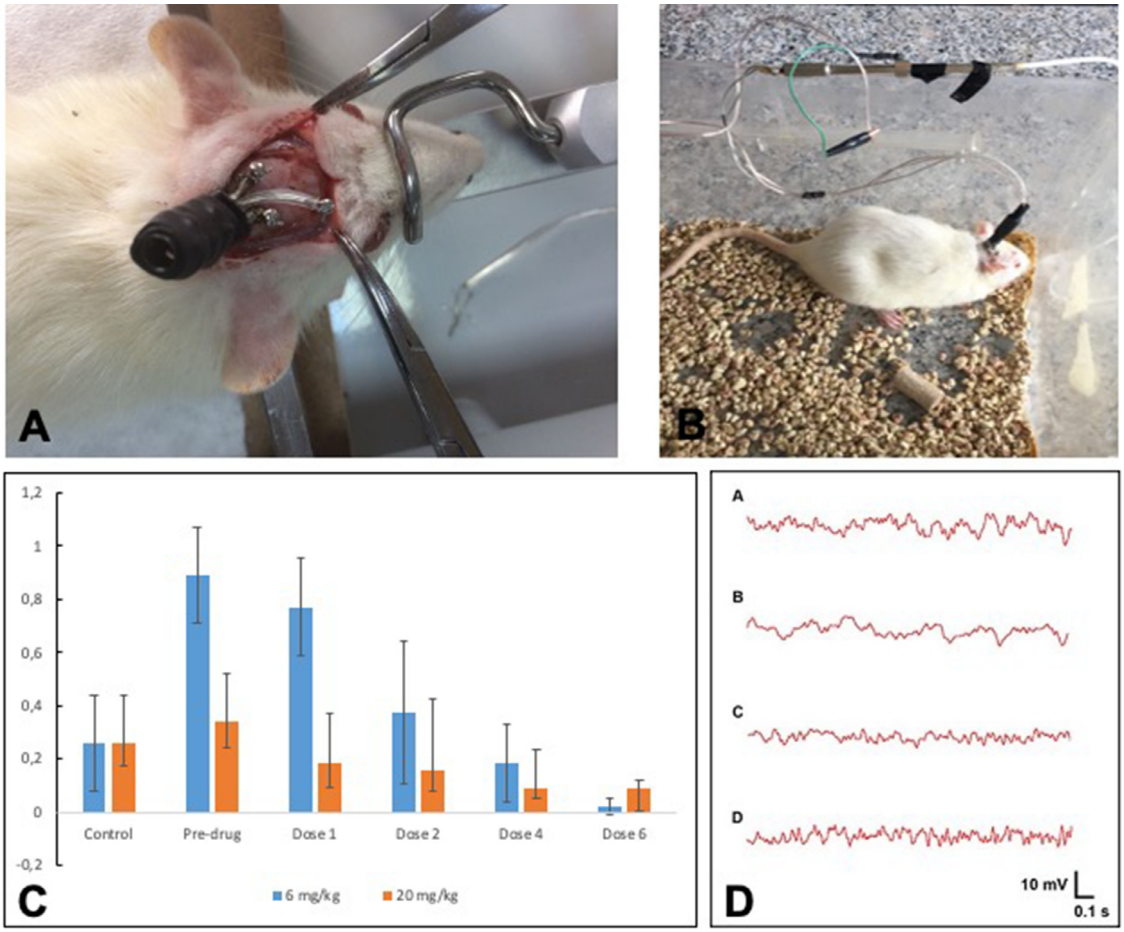


Fig. 6. EEG electrode placement in cranium by drilling holes (**A**). Appearance of rats during EEG monitoring (**B**). Effect of 6 mg/kg and 20 mg/kg LCM treatments on EEG Delta SPD values. It is observed that SPD values were close to control group after the 4th dose and 1st dose of 6 mg and 20 mg/kg LCM treatment respectively (**C**). Delta waves for each group (**D**). **a-** Control group. **b-**Trauma group **c-** EEG waves of 6mg/kg LCM treated group. **d-** EEG waves of 20 mg/kg LCM treated group. After LCM treatment, the strength of the delta waves decreased with dose-dependent manner. The similarity of delta waves in high-dose LCM treatment group and control group is remarkable.

activation of central nervous system (CNS) immune cells, cerebral infiltration of peripheral immune cells, and upregulation of inflammatory cytokines, chemokines, and reactive oxygen species [21]. The key to developing effective therapies for TBI is to better understand and identify the precise mechanisms. **For this purpose, experimental animal models have been developed, and rodents (mice, rats, rabbits, etc.) are widely used in TBI models created by reducing the weight. The rodent brain volume provides convenience for the experimental investigation as TBI is the specific focal injury model. Because, the weight (metal disk) is focally dropped to a single point of skull, and since that weight-drop model is carried out in a controlled manner, it is advantageous in terms of determining the region to be examined [22,23].** However, despite numerous experimental studies reporting promising results, a pharmacological treatment that demonstrated clinical efficacy following TBI has still not been reported. The present study was designed to investigate the effects of LCM treatment on post-TBI structural damage and EEG monitoring.

LCM was synthesized as a member of a family of functionalized amino acids for anticonvulsant properties [4,24]. It selectively enhances slow inactivation of voltage-gated sodium channels and interacts with CRMP-2 which is one of the major phosphoproteins in the developing nervous system and involved in neuronal differentiation and axonal outgrowth [4,25]. Preclinical and clinical studies have demonstrated that LCM protects against generalized seizures, complex partial-onset seizures, and status epilepticus [5,6,26,27,28]. In addition, it has demonstrated analgesic activity in various animal models [7,29]. Apart from this, LCM has demonstrated potent effects in animal models for a variety of CNS disorders like schizophrenia and stress induced anxiety [30]. Besides all these effects, it is a question of whether LCM has a neuroprotective effect. Although its neuroprotective effect has been demonstrated in hypoxic and hyperoxic brain injury models in rats [9,11], its neuroprotective effect in TBI is still unclear. In our english literature review, we can found only two experimental studies investigating the neuroprotective effect of LCM after TBI [8,10]. Wang et al. treated the mices with either low-dose (6 mg/kg) or high-dose (20 mg/kg) LCM after closed head injury. As a result, authors reported that, high dose LCM was associated with improved functional outcome. Also, LCM reduces histological evidence of acute neuronal injury and neuroinflammation such as neuronal loss and microglial activation [10]. However, Pitkänen et al. treated the adult rats with 30 mg/kg LCM after lateral fluid-percussion injury. As a result; authors stated that; acute LCM treatment did not improve post-TBI cognitive recovery or motor recovery. Also LCM treatment did not result in any protection against axonal or neuronal injury after traumatic brain injury in rats [8]. These two valuable studies reported contrasting results, leading to confusion. Therefore, we investigated whether LCM has a neuroperotective effect after TBI **on the 3rd dayof experiment. We choosed to evaluate the effects of LCM by ending our experiment on 3rd day because**

of neuroplasticity. Until the 1900s, the neuronal regeneration in the brain was considered to stop in adulthood, but then this view has changed as a results of the studies of many researches. Especially between the 1960s and 1990s, the neuroplasticity began to gain acceptance. The presence of neuroplasticity has been demonsrated in the hypothalamus, hippocampus, dentate gyrus and olfactory bulb regions in rodents, such as rats. Whereas how long neuroplasticity takes place is still controversial, it takes a long time in adults [31,32]. Therefore, in our study we planned to terminate the experiment on the 3rd day, in order to reveal the effects of LCM in the short term after TBI before neuroplasticity begins. The key findings of current studies were; 1- Apoptotic cells with pyknotic nuclei and HIF-1α positive cells were decreased after LCM treatment with dose dependent manner. 2- VEGF and Neu N positive cells were increased after LCM treatment with dose dependent manner. This histological findings could be interpreted in the following manner: LCM treatment accelerates the recovery of brain damage by stimulating the formation of new vessel which prevent the hypoxia and apoptosis. 3- On the one hand serum level of MDA (an oxidative stress marker) decreased, on the other SOD (anti-oxidant enzyme) increased with LCM treatment. **There is no data in the literature about the antioxidant effect of LCM in the TBI model. Only, in rats with spinal cord injury (SCI), LCM administration (30 mg/kg, 8 h intervals) enhanced the the SOD level compared to non-treated groups. While the SOD level was detected as 0,20 ± 0,038 ng/mL in sham group, it was 0,84 ± 0,234 ng/mL in SCI group treated with LCM [33]. The fact that, the SOD level was higher in LCM groups than the control or sham groups suggested that the antioxidant property of LCM was considerably effective.**

Existing research findings have suggested that oxidative stress can disturb the balance of electrical activity in the brain. Delta band is considered a marker of brain sufferance or pathological condition. Indeed, several studies found that slow wave activity, particularly in the range of delta frequencies marks pathological brain abnormality resulting from neurological damage, such as cerebral infarct, contusion, local infection, tumor or subdural hematoma [34,35]. Additional evidence of the link between slow delta activity and brain damage arose from studies of neurological or psychiatric patients affected by Alzheimer's disease, mild cognitive impairment, aphasia, dyslexia, schizophrenia or depression [36,37]. In this study, we recorded all rats to determined whether acute LCM treatment affect the slow wave activity by EEG monitoring. Trauma and drug dose-related changings were more dramatic in the low frequency bands, particularly in the delta band. While the strength of the delta wave was highest in TBI group, it decreased after LCM treatment with dose-dependent manner. The similarity of delta waves in high-dose LCM treatment group and control group were remarkable.

Conclusion

No existing study has produced results suggesting that different doses of LCM has therapeutic effect against TBI, using EEG recording in addition to histological and biochemical evaluations in rats. The present study revealed that LCM, an anti-epileptic agent, has prominent neuroprotective effects against TBI because of its anti-apoptotic, and antioxidant activities when administered immediately after trauma in repeated doses.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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