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Biochemical, pathological and ultrastructural investigation of whether lamotrigine has neuroprotective efficacy against spinal cord ischemia reperfusion injury

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ABSTRACT

Introduction: Lamotrigine, an anticonvulsant drug with inhibition properties of multi-ion channels, has been shown to be able to attenuates secondary neuronal damage by influencing different pathways. The aim of this study was to look into whether lamotrigine treatment could protect the spinal cord from experimental spinal cord ischemia-reperfusion injury.

Materials and methods: Thirty-two rats, eight rats per group, were randomly assigned to the sham group in which only laparotomy was performed, and to the ischemia, methylprednisolone and lamotrigine groups, where the infrarenal aorta was clamped for thirty minutes to induce spinal cord ischemia-reperfusion injury. Tissue samples belonging to spinal cords were harvested from sacrificed animals twenty-four hours after reperfusion. Tumor necrosis factor-alpha levels, interleukin-1 beta levels, nitric oxide levels, superoxide dismutase activity, catalase activity, glutathione peroxidase activity, malondialdehyde levels and caspase-3 activity were studied. Light and electron microscopic evaluations were also performed to reveal the pathological alterations. Basso, Beattie, and Bresnahan locomotor scale and the inclined-plane test was used to evaluate neurofunctional status at the beginning of the study and just before the animals were sacrificed.

Results: Lamotrigine treatment provided significant improvement in the neurofunctional status by preventing the increase in cytokine expression, increased lipid peroxidation and oxidative stress, depletion of antioxidant enzymes activity and increased apoptosis, all of which contributing to spinal cord damage through different paths after ischemia reperfusion injury. Furthermore, lamotrigine treatment has shown improved results concerning the histopathological and ultrastructural scores and the functional tests.

Conclusion: These results proposed that lamotrigine may be a useful therapeutic agent to prevent the neuronal damage developing after spinal cord ischemia-reperfusion injury.

Introduction

During the thoracoabdominal aortic surgery, cessation and restoring of spinal cord blood flow can cause initially ischemic and subsequently reperfusion injury on the neuronal structures which is called the ischemia/reperfusion injury of spinal cord (SCIRI) [1]. Paraplegia as a result from SCIRI results in physically, socially and financially deprived victims [2]. SCIRI is a composite of interwoven pathological pathways result in apoptotic cell death which includes decreases of cellular energy production, mitochondrial dysfunction, alteration of ionic distribution, loss of membrane potential, depolarization of cellular membranes, extreme discharge of glutamate,

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alterations of glutamate receptor activations and glutamate transporter expression, increase in oxidative stress and inflammation [3,4]. Despite various pharmacological agents have been studied to prevent these interconnected pathological processes and enhance functional outcome after SCIRI [5-16], no neuroprotective treatment has been successfully effective so far.

Lamotrigine (LTG) is an anticonvulsant drug mainly used for the treatment of epilepsy and bipolar disorders. It has been showed that LTG has protective properties on neuronal tissues in different laboratory studies, such as hypoxic-ischemic encephalopathy, cerebral ischemia, subarachnoid hemorrhage and traumatic spinal cord injury, mainly through presynaptic inhibition of glutamate release [17-21]. Herein, we intended to look into neuroprotective effects of LTG on biochemical, histological, ultrastructural and behavioral parameters in a rat model of SCIRI. No studies reporting the neuroprotective actions of LTG on SCIRI had been found in the literature.

Materials and methods

Drug treatments and study groups

All experimental procedures used in this study were approved by the Ministry of Health Ankara Education and Research Ethics Committee in accordance with the European Communities Council Directive (86/609 / EEC) dated November 24, 1986 on the protection of experimental animals. Lamotrigine (LTG; lamotrigine isethionate, GlaxoSmithKline, Istanbul, Turkey) (20 mg/kg/day) intraperitoneally was used in this study and was dissolved in distilled water in the ultrasonic bath (Bandelin-Sonorex, BK100H, Berlin, Germany). The administration time, route and dosage of LTG with its solvent were determined according to previous studies [17-25]. Anesthesia induction was achieved via intraperitoneal injection of 50 mg/kg ketamine HCl (Ketalar®; Pfizer Inc., USA), and 10 mg/kg xylazine HCl (Rompun 1 2%; Bayer HealthCare AG, Germany).

Thirty-two adult male Wistar Albino rats weighing 250±20 g were assigned to four groups randomly, with each group consisting of eight rats.

The description of groups was as follows:

- **Group 1:** sham (n = 8); rats underwent only a simple laparotomy without aortic occlusion after dissection. After sacrificing of the animals at the 24th hours of injury, nonischemic samples of spinal cord were taken to elucidate normal morphology and biochemical outcomes.
- **Group 2:** ischemia (n = 8); rats suffering from SCIRI injury received a single dose of 2 ml of distilled water into the intraperitoneal space as vehicle equal to the LTG volume.
- **Group 3:** methylprednisolone (MP) (n = 8); MP (Prednol, Mustafa Nevzat, Turkey) was administered into the intraperitoneal space of rats at a single 30 mg/kg dose just after induction of ischemia-reperfusion injury [9,16].
- **Group 4:** LTG (n = 8); as in group 3, but, 30 minutes before the abdominal cavity was opened, LTG was administered intraperitoneally at a dose of 20 mg/kg for a single time [17-25].

No drug reaction came out in the study groups.

Surgical procedure

The rats were kept under suitable humidity (65–70%) and temperature (23 ± 2°C), with free access to food and water, under a twelve-hour dark and light cycle.

Rats with rectal probes were placed on the heating pad which maintains the body temperature of 37°C, following the induction of anesthesia which applying with intraperitoneally 10 mg/kg xylazine (Rompun, Bayer, Turkey) and 50 mg/kg ketamine (Ketalar, Parke Davis, Turkey) with allowing spontaneous respiration.

Spinal cord ischemia-reperfusion was performed in accordance with the time and method to create sufficient damage as previously described [25,26]. After entering the abdominal cavity transperitoneally, aorta was exposed from the beginning of the left renal artery proximally to the bifurcation point distally, where it would be clamped. Immediately after injection of 200 IU/kg heparin, the aorta is clamped with clamps with a closing force of 70 g (Yasargil FE 721; Aesculap, Tuttlingen, Germany) at the two points mentioned above to create ischemia, and it is visually confirmed that the femoral artery pulse disappears during the 30-minute ischemia period and returns during the reperfusion period. After 24h reperfusion period, the rats were sacrified with injection of 200 mg/kg pentobarbital (Nembutal; Oak Pharmaceuticals, Lake Forest, IL), and spinal cord segments from L4 to L6 levels were removed and divided into 3 equal parts for examination of light microscopy (proximal part), electron microscopy (middle part) and biochemical parameters (caudal part). Tissues to be used in biochemical analysis were stored at -80°C till the analysis.

Biochemical procedures

After homogenization with physiological saline solution, tissues were centrifuged at 4000 rpm for 20 minutes to obtain supernatants from the tissues to be used in analysis.

Tissue tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) analysis

ELISA kits (Uscn Life Science Inc., Wuhan, China), working according to the instructions for use and expressed as U/g-protein, tested tissue TNF-α and IL-1β levels, which are indicators of inflammation.

Tissue nitric oxide (NO) analysis

Tissue NO levels were measured as described by Miranda et al. [27]. In this method, proteins of tissues homogenized in saline solution are precipitated using ethanol. The substances are allowed to be separated for fifteen minutes at 25°C to recover the supernatant. 0.5 ml supernatant, 0.5 ml vanadium (III) chloride (8 mg VCl_3/ml) and 0.5 ml freshly prepared Griess reagent (1% sulfanilamide, 2% phosphoric acid and 0.1% N-1 naphthylethylene diamine dihydrochloride; 500 µl) mixture is incubated at 37°C for 30 minutes by vortexing. Then, the absorption at 540 nm is measured using a dual beam spectrophotometer and the results are expressed as nmol/mg-protein.

Tissue glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) analysis

ELISA kits (Uscn Life Science Inc., Wuhan, China), working according to the instructions for use and expressed as U/g-protein, tested levels of tissue GPx and CAT activities, which are indicators of oxidative stress. Total (Cu - Zn and Mn) SOD (EC 1.15.1.1) activity of the supernatant in the ethanol phase was determined according to the description of Sun et al. [28]. After adding 1.0 ml of ethanol/chloroform mixture (5/3, v/v) to the same volume of sample and centrifuging, the amount of enzyme that caused 50% inhibition of nitrobluetetrazolium reduction was defined as one unit of SOD and the enzyme activity was expressed as U/mg-protein.

Tissue malondialdehyde (MDA) analysis

Tissue MDA levels were assessed by a method based on reaction with thiobarbituric acid (TBA) as previously described by Ohkawa et al [29]. 375 µl acetic acid (pH 3.5, 20%) and 375 µl TBA (0.6%) are added to a mixture of 100 ml of tissue homogenate and 50

ml of sodium dodecyl sulfate (SDS, 8.1%) which is vortexed and incubated at room temperature. After this entire mixture is heated in boiling water for 60 minutes and subsequently cooled to room temperature, 1.25 ml of butanol: pyridine (15: 1) is added to each test tube. Then this vortexed mixture is centrifuged at 4000 g for 5 minutes and the absorption of 750 µl organic layer is read at 532 nm in 1 milliliter cells. MDA concentrations were expressed as nmol/mg-protein.

Tissue caspase-3 analysis

ELISA kits (Uscn Life Science Inc., Wuhan, China), working according to the instructions for use and expressed as U/g-protein, tested level of caspase-3 activity, which is an indicator of apoptosis.

Histopathological procedures

Light microscopy examination

A histopathologist who blinded to the study design evaluated hematoxylin-eosin (H&E) stained spinal cord tissue sections with a thickness of 5 µm by using a light microscopy. A semi-quantitative scoring system scored neuronal degeneration, cellular edema, hemorrhage, congestion and inflammation in spinal cord tissue sections as follows; 0: absent; 1: mild; 2: moderate; and 3: common. The average of the scores of these four different parameters determined the pathological score for each spinal cord tissue [30].

In order to more detailed grading of neuronal injury; spinal cord anterior horn motor neurons were counted in three segments for all rats and averaged [31].

Transient electron microscopy examination

Semi-thin sections with a thickness of 2 µm stained with methylene blue and ultra-thin sections with a thickness of 60 nanometers stained with uranyl acetate and lead citrate were examined using a Nikon Optiphot (Nikon Corporation, Tokyo, Japan) light microscopy and transmission electron microscopy Jeol JEM 1200 EX (Jeol Ltd., Tokyo, Japan), respectively. As described by Kaptanoğlu et al. [32], every 100 large diameter myelinated axons, medium diameter myelinated axons, and small diameter myelinated axons were counted, evaluated, and scored between 0 and 3, as follows: 0 = ultrastructurally normal myelinated axon, 1 = separation in myelin configuration, 2 = interruption in myelin configuration, and 3 = honeycomb appearance in myelin configuration.

Neurological evaluation

Basso, Beattie and Bresnahan (BBB) locomotor scale and inclined plane test evaluated the neurofunctional status of rats at the beginning of the experiment and just before sacrification. In the BBB test rats were scored between 0 and 21 points from no activity to normal activity [33], while in the inclined plane test the angle at which the rats stood at the maximum slope for 5 s was recorded by an independent observer for evaluation of motor function [34].

Statistical analysis

Data analysis was performed by using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). Whether the distributions of continuous variables were normally or not was assessed by Shapiro Wilk test. Levene test was used for the evaluation of homogeneity of variances. Data were shown as mean ± standard deviation or median (IQR), where applicable.

While the mean differences among groups were analyzed by using One-Way ANOVA, otherwise, Kruskal Wallis test was applied for comparing the median values. When the p value from One-Way ANOVA or Kruskal Wallis test statistics are statistically significant post hoc Tukey HSD or Conover's non-parametric multiple comparison test were used to know which group differ from which others. A p value less than 0.05 was considered statistically significant.

Results

Tissue tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) analysis

Spinal cord ischemia-reperfusion injury leads to produce TNF-α and IL-1β by macrophages and neutrophils at the time of the initial ischemia and subsequent reperfusion. Therefore, statistically significant elevation was observed on cytokine levels in the ischemia group when compared to the sham group ($p < 0.001$ for both comparisons). An increase of proinflammatory cytokines was also observed in both the LTG and the MP groups compared with the sham group ($p < 0.05$ and $p < 0.01$, respectively). However, both LTG and MP treatments provided marked decrease in the tissue TNF-α and IL-1β levels when compared to the ischemia group ($p<0.05$ for both comparisons). No such difference was observed with respect to tissue TNF-α and IL-1β levels between the MP and the LTG groups ($p = 0.857$ for both comparisons) (Fig. 1A and 1B).

Tissue nitric oxide (NO) analysis

Spinal cord ischemia-reperfusion injury results in elevation of NO levels due to free radical production, which is an indicator of the oxidative stress. Whence, tissue NO levels of the sham group were significantly lower compared to the ischemia, MP and LTG groups ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively). Tissue NO levels increased significantly in the MP and the LTG groups compared to the ischemia group ($p < 0.05$ for both comparisons). There was no statistical difference between the treatment groups ($p = 0.622$) (Fig. 1C).

Tissue glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) analysis

Spinal cord ischemia-reperfusion injury can cause impairment of antioxidant defense mechanisms under highly elevated oxidative stress. Therefore, tissue antioxidant enzyme activities (GPx, CAT and SOD) in the treatment groups (MP and LTG) were significantly higher than in the ischemia group ($p < 0.05$, for all comparisons). There was no difference in tissue GPx, CAT and SOD activities between the treatment groups ($p = 0.993$, $p = 0.928$ and $p = 0.447$, respectively) (Fig. 1D,E,F).

Tissue malondialdehyde (MDA) analysis

Spinal cord ischemia-reperfusion injury can lead to an elevation of MDA levels due to lipid peroxidation. Whence, tissue MDA levels of the sham group were significantly lower compared to the ischemia group and the MP and the LTG groups ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively). Similarly, tissue MDA levels of both treatment groups were significantly lower compared to the ischemia group ($p < 0.05$ for both). The treatment groups did not differ among themselves with regard to tissue MDA levels ($p = 0.788$) (Fig. 1G). These results suggest that both MP and LTG treatments significantly attenuated the elevated MDA levels due to SCIRI, and protected the spinal cord from lipid peroxidation.

Tissue caspase-3 analysis

The increase in tissue caspase-3 activity due to apoptotic cell death after SCIRI resulted in the detection of lower mean tissue

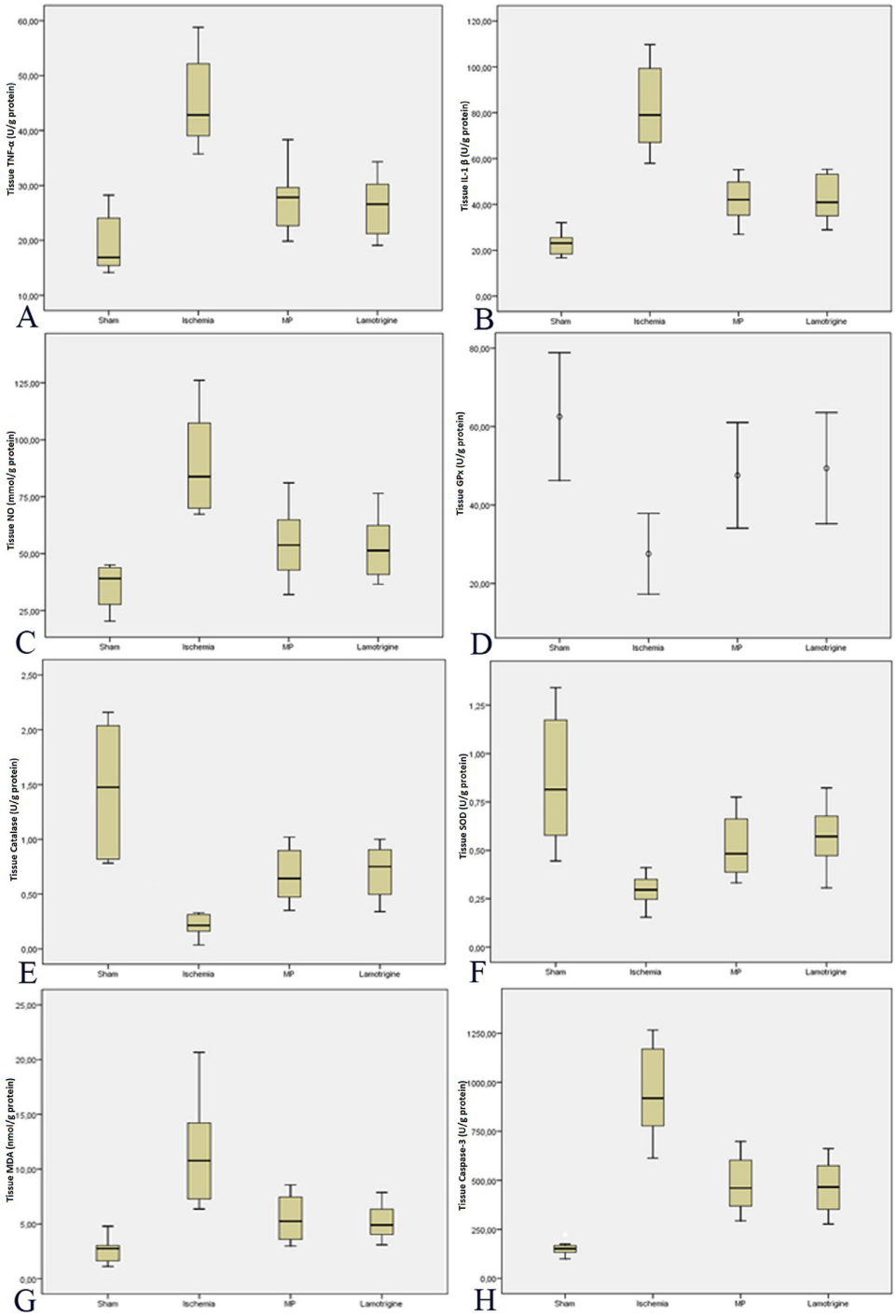

Fig. 1. A, **B, C, E, F, G, H.** The horizontal lines in the middle of each box indicates the median, while the top and bottom borders of the box mark the 25th and 75th percentiles, respectively. The whiskers above and below the box mark indicates the maximum and minimum levels. **Fig. 1D.** The box in the middle of each whiskers indicates the arithmetic mean, while the whiskers above and below the box mark the + SD and – SD levels, respectively. CAT: catalase, GPx: glutathione peroxidase, IL-1β: interleukin-1 beta, MDA: malondialdehyde, MP: methylprednisolone, NO: nitric oxide, SOD: superoxide dismutase, TNF-α: tumor necrosis factor-alpha.

caspase-3 activity in the sham group compared to the ischemia group and the MP and the LTG groups ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively). However, the average tissue caspase-3 activities were lower in both treatment groups compared to the ischemia group ($p < 0.05$ for both comparisons). There was no such difference between the MP and the LTG groups ($p = 0.822$) (Fig. 1H). These results clearly demonstrate that the ischemia-induced elevation in tissue caspase-3 activity was largely attenuated by LTG treatment in post-ischemia period.

All biochemical results were summarized in Table 1.

Histopathological examination

The histological scores of the study groups regarding neuronal degeneration, cellular edema, hemorrhage/congestion, and inflammation are represented in Table 2.

Light microscopic examination showed the regular morphology of spinal cord samples in the sham group (Fig. 2A). In the

Table 1
Biochemical measurements relevant to the study groups.

Variables	Sham	Ischemia	MP	LTG	p-value
Tissue TNF-α (U/g protein)	16.87 (10.17)[a,b,c]	42.83 (13.38)[a,d,e]	27.83 (8.04)[b,d]	26.57 (9.81)[c,e]	**<0.001**
Tissue IL-1β (U/g protein)	23.10 (7.84)[a,b,c]	79.02 (39.17)[a,d,e]	42.11 (17.59)[b,d]	40.94 (19.03)[c,e]	**<0.001**
Tissue NO (mmol/g protein)	39.02 (17.12)[a,b,c]	83.76 (40.53)[a,d,e]	53.72 (25.21)[b,d]	51.36 (26.41)[c,e]	**<0.001**
Tissue GPx (U/g protein)	62.55±16.31[a]	27.55±10.30[a,d,e]	47.56±13.49[d]	49.39±14.18[e]	**<0.001**
Tissue CAT (U/g protein)	1.47 (1.23)[a,b,c]	0.21 (0.17)[a,d,e]	0.64 (0.50)[b,d]	0.75 (0.48)[c,e]	**<0.001**
Tissue SOD (U/g protein)	0.81 (0.69)[a,b,c]	0.30 (0.12)[a,d,e]	0.48 (0.34)[b,d]	0.57 (0.26)[c,e]	**<0.001**
Tissue MDA (nmol/g protein)	2.76 (1.42)[a,b,c]	10.77 (7.86)[a,d,e]	5.24 (4.19)[b,d]	4.90 (2.83)[c,e]	**<0.001**
Caspase-3 (U/g protein)	151.46 (41.74)[a,b,c]	918.51 (429.74)[a,d,e]	460.59 (252.20)[b,d]	465.21 (238.45)[c,e]	**<0.001**

CAT: catalase, GPx: glutathione peroxidase, IL-1β: interleukin-1 beta, LTG: lamotrigine, MDA: malondialdehyde, MP: methylprednisolone, NO: nitric oxide, SOD: superoxide dismutase, TNF-α: tumor necrosis factor-alpha.
[a] Sham vs Ischemia ($p < 0.001$),
[b] Sham vs MP ($p < 0.01$),
[c] Sham vs LTG ($p < 0.05$),
[d] Ischemia vs MP ($p < 0.05$),
[e] Ischemia vs LTG ($p<0.05$).

Table 2
Histopathological parameters relevant to the study groups.

Variables	Sham	Ischemia	MP	LTG	p-value
Edema	0 (0-0)[a,b,c]	2 (2-3)[a,d,e]	1 (0-2)[b,d]	1 (0-2)[c,e]	**<0.001**
Congestion	0 (0-0)[a,b,c]	2 (2-3)[a,d,e]	1 (1-2)[b,d]	1 (1-1)[c,e]	**<0.001**
Inflammation	0 (0-0)[a,b,c]	1 (1-1)[a]	1 (0-1)[b]	1 (0-1)[c]	**<0.001**
Degeneration	0 (0-0)[a,b,c]	2 (2-3)[a,d,e]	1 (1-2)[b,d]	1 (1-1)[c,e]	**<0.001**
Pathological score	0 (0-0)[a,b,c]	7 (7-9)[a,d,e]	4 (3-6)[b,d]	4 (3-5)[c,e]	**<0.001**
Number of normal neurons	46.5±4.34[a,b,c]	21.5±2.33[a,d,e]	36.0±2.83[b,d]	36.7±2.37[c,e]	**<0.001**

LTG: lamotrigine, MP: methylprednisolone.
[a] Sham vs Ischemia ($p < 0.001$),
[b] Sham vs MP ($p < 0.001$),
[c] Sham vs LTG ($p < 0.001$),
[d] Ischemia vs MP ($p < 0.01$),
[e] Ischemia vs LTG ($p < 0.001$).

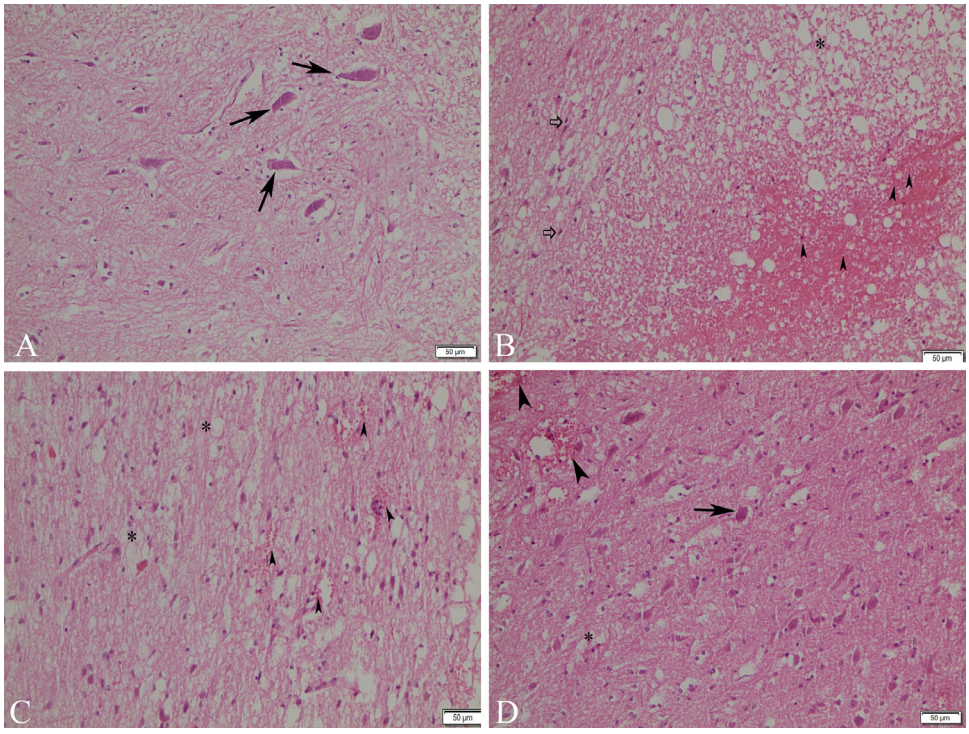

Fig. 2. Photomicrographs of 5-mm-thick spinal cord tissue sections from the different treatment groups (H&E, X20). **A;** Sham group showing normal spinal cord parenchyma with normal-appearing neurons (filled arrow). **B;** Ischemia group showing diffuse hemorrhage and congestion (arrowhead), and widespread edema (asterix) with highly degenerated neurons (arrow) in the gray matter. **C;** MP group, showing mild edema (asterix) and mild hemorrhagic congestion (arrowhead). **D;** LTG group, showing mild edema (asterix) and mimimal congestion (arrowhead) with less degenerated normal neurons (arrow).

spinal cord samples of ischemia group, there were severe vascular congestion, pericellular and interstitial edema consistent with the ischemia-reperfusion injury. There was also pyknosis, intense swelling of axonal structures, loss of cytoplasmic features, and cytoplasmic eosinophilia indicating neuronal injury (Fig. 2B). These pathological alterations were considerably reduced in the spinal cord samples of MP group as well as LTG group (Fig. 2 C and D).

The pathological scores of ischemia group statistically higher than the sham group ($p < 0.001$). There were also higher pathological scores in both the MP and the LTG groups compared with the sham group ($p<0.001$ for both comparisons). However, both the MP and LTG groups demonstrated significantly lower pathological scores than the ischemia group ($p < 0.01$ and $p < 0.001$, respectively). No significant difference was observed between the MP and LTG groups in terms of pathological scores ($p = 0.248$).

The count of normal motor neurons within the anterior horns of the spinal cord was significantly greater in the sham group than in the other groups which exposed to ischemia reperfusion injury ($p<0.001$ for all comparisons). Also, ischemia group showed statistically significantly lower normal motor neurons number than either MP or LTG groups ($p < 0.001$ for both comparisons). This difference was not statistically significant between the MP and LTG groups ($p = 0.961$) (Table 2).

Ultrastructural examination

In TEM examination of the sham group; the ultrastructure of the neurons was normal in appearance, and there were no pathological changes in the intracellular organelles, nuclei, membranes and perineuronal tissues. Nevertheless, mild separations were observed in a very few of the large-sized myelinated axons in a small part of the myelin sheath which could be due to delayed fixation. The ultrastructural features for the rest of the myelinated axons in all size were found to be normal (Fig. 3A).

In the ischemia group; severe ultrastructural pathological changes were observed in both white and gray matter due to SCIRI, including the interruptions and separations of myelin sheats in white matter axons, and swollen mitochondria, intrastoplasmic neuronal vacuoles and perineural edema in the gray matter (Fig. 3B).

TEM examinations of tissue samples in the MP group revealed perineural edema with swollen mitochondria and vacuoles in the neuron cytoplasm in gray matter as ultrastructural pathological changes. Additionally; mostly large- and medium-sized myelinated axons with a few small-sized myelinated axons showed separations in myelin configurations in white matter (Fig. 3C).

Perinuclear cisternal dilatations with a small amount of perineural edema have been observed in TEM examination of the gray matter of the LTG group tissue samples. Separations in myelin configuration were observed in this group especially in most large-sized and medium-sized myelinated axons in contrast to majority of the small-sized myelinated axons were ultrastructurally normal (Fig. 3D).

Small-, medium- and large-sized myelinated axons were more injured in the ischemia group compared to the sham group ($p < 0.01$ for all comparisons). Similarly, these differences were statistically significant with regard to myelin damage for small-, medium- and large-sized myelinated axons between the MP and the ischemia group ($p < 0.01$ for all comparisons). The difference was also statistically significant between LTG group and the ischemia group in terms of damage to small-, medium- and large-sized myelinated axons ($p < 0.01$ for all comparisons). These results suggest that both LTG and MP treatments protected the small-, medium- and large-sized myelinated axons effectively from I/R injury.

The electron microscopy results summarized in Table 3.

Neurological evaluation

The mean values of neurologic examination scores in different groups were summarized in Table 4.

Neurological scores were significantly deteriorated in the ischemia group compared with the sham group in terms of BBB scores and mean angles recorded in the inclined-plane test ($p < 0.001$ for both comparisons). Both LTG and MP groups showed higher BBB scores and the angles in the inclined-plane test when compared to the ischemia group ($p < 0.001$ for both comparisons). The BBB scores and the mean angles recorded in the inclined-plane test had not been shown a difference of statistical significance between the MP group and the LTG group ($p = 0.560$ and $p = 0.995$, respectively). Therefore, we suggested that LTG treatment could be beneficial to ameliorate functional neurological deficit after SCIRI as well as MP.

Discussion

One of the key pathological changes in the spinal cord that occur during the prolonged aortic occlusion is massive release of excitatory amino acid neurotransmitters, notably glutamate. Excessive accumulation of glutamate in the synaptic cleft may result in cell swelling, vacuolization, apoptosis and neuronal death in the course of SCIRI through leading to over-stimulation of glutamate receptors and the disruption of glutamate transporters. Thus, a trend towards ameliorating excitotoxic neuronal damage by modifying excitatory amino acid release, excitatory amino acid receptor antagonism and subsequent inhibition of proteolysis and lipid peroxidation has potentially emerged [35-44].

The results of pharmacological research reveal that LTG prohibits the aberrant extracellular accumulation of glutamate as well as the action potential discharges caused by glutamate [45]. Additionally, LTG has been suggested to inhibit vesicular release of glutamate by inhibiting high voltage activated calcium currents [46]. Subsequently, LTG is a blocker for voltage-gated sodium channels, depending on the dose [47-49]. Besides all these pharmacological properties, recent experimental studies suggested that LTG had protective effects through suppressing the inflammatory response, decreasing the oxidative stress and inhibiting the apoptosis, against the cellular damage in ischemia reperfusion injury [50-52]. However, to best of our knowledge, protective effects of LTG against neuronal damage in SCIRI had not been studied before.

Prolonged ischemia triggers an early inflammatory response due to releasing of inflammatory cytokines like TNF-α and IL-1β by residential microglia and macrophages in severely injured ischemic cord. During reperfusion, many neutrophils, macrophages and microglial cells migrate to the damaged area, result in amplification of initial inflammatory response through reactions mediated by chemoattractrants and cytokines [53,54]. High levels of extracellular glutamate act as chemoattractant and causes the infiltrating cell accumulation including microglia, neutrophils and macrophages into the injured tissue by activating ionotrophic class 1 metabotropic glutamate receptors [55]. Activation of ionotropic glutamate receptors leads to an enhancement in the release of TNF-α, and IL-1β mainly by the neutrophil's azurophilic granules, which subsequently result in edema, hemorrhage and apoptotic cell death [56,57]. Therefore, high TNF-α, and IL-1β levels are reliable markers of inflammation during SCIRI. Additionally, previous studies showed that LTG had anti-inflammatory and immunomodulatory actions via inhibition of TNF-α, IL-1β and IL-2 secretions [58]. In consistently, we demonstrated that ischemia-reperfusion injury leads to marked elevation of TNF-α and IL-1β levels and these alterations were significantly decreased by both LTG and MP administration, disclosing the anti-inflammatory effects of both drugs.

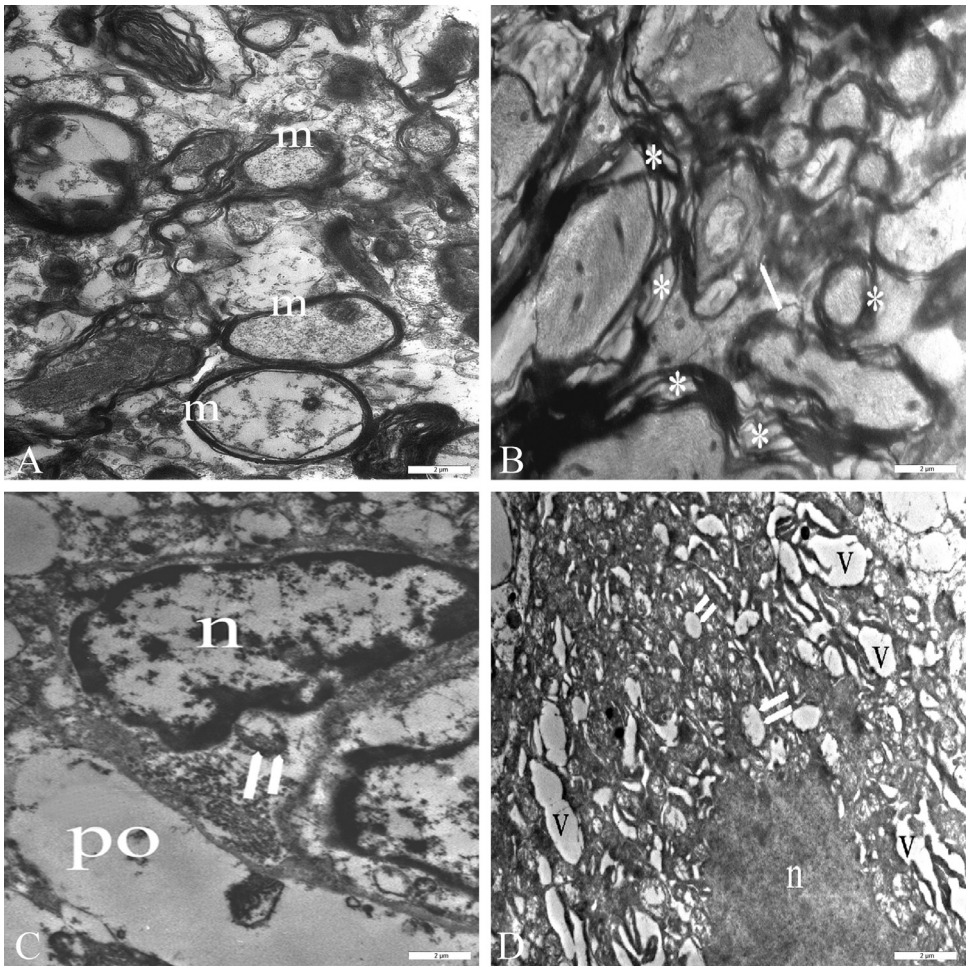

Fig. 3. Transmission electrom microscopy of the groups. **A.** Transmission electron microscopic examination of the tissue samples of the sham group showed normal spinal cord ultrastructure in the gray and white matters. The mild seperations in the myelin configuration observed in a very few of the large-sized myelinated axons may be explained by delayed fixation **m**: ultrastructurally normal myelinated axon (Bar represents 2 µm). **B.** Electron micrograph from the ischemia group showing separations in myelin configuration (*) in small, medium, large sized myelinated axons and interruption in myelin configuration (arrow) in a medium sized myelinated axon (orginal magnification x 5000). **C.** Electron micrograph of the MP group showing perineural edema **(po)** with swollen mitochondria **(double arrow) n**: nucleus of neuron **D.** Electron micrograph of the LTG group showing vacuoles (**v**) and a swollen mitochondria (double arrows) inside the cytoplasm of neurons. **n**: nucleus of neuron.

Table 3
Electron microscopy results.

Myelinated Axon	Sham	Ischemia	MP	LTG	p-value
Small-sized	0 (0-0)[a,b,c]	115 (110-120)[a,d,e]	30 (26-32)[b,d]	33 (31-37)[c,e]	**<0.001**
Medium-sized	0 (0-0)[a,b,c]	140 (128-144)[a,d,e]	91 (87-93)[b,d]	86 (79-90)[c,e]	**<0.001**
Large-sized	4 [2-6][a,b,c]	169 (167-180)[a,d,e]	115 (113-119)[b,d,f]	94 (91-97)[c,e,f]	**<0.001**

LTG: lamotrigine, MP: methylprednisolone.
[a] Sham vs Ischemia ($p < 0.01$),
[b] Sham vs MP ($p < 0.01$),
[c] Sham vs LTG ($p < 0.01$),
[d] Ischemia vs MP ($p < 0.01$),
[e] Ischemia vs LTG ($p < 0.01$),
[f] MP vs LTG ($p < 0.01$).

Table 4
The results of neurological assessment of the study groups.

Myelinated Axon	Sham	Ischemia	MP	LTG	p-value
BBB	21.0 (0.00)[a,b,c]	1.5 (1.00)[a,d,e]	8.0 (2.75)[b,d]	8.5 (3.00)[c,e]	**<0.001**
Inclined plane angel	78.0±4.14[a,b,c]	37.5±4.98[a,d,e]	60.7±7.24[b,d]	61.5±7.69[c,e]	**<0.001**

LTG: lamotrigine, MP: methylprednisolone.
[a] Sham vs Ischemia ($p < 0.001$),
[b] Sham vs MP ($p < 0.001$),
[c] Sham vs LTG ($p < 0.001$),
[d] Ischemia vs MP ($p < 0.001$),
[e] Ischemia vs LTG ($p < 0.001$).

Reactive oxygen species, which plays a fundamental role in immune system regulation and maintenance of redox homeostasis under physiologic conditions, plays a detrimental role due to overproduction. During the ischemia reperfusion large amount of reactive oxygen species expose due to membrane depolarization and inflammatory reactions. Oxidative balance cannot be maintained between the reduced expression of antioxidant enzymes such as SOD, CAT and GPx and the increased production of reactive oxygen species. This situation called "oxidative stress" [59,60]. Additionally, excessive extracellular glutamate accumulation during ischemia contributes to increased intracellular level of calcium which resulting in an increase in NO, which leads to DNA damage by activation of calcium-dependent proteases and nitric oxide synthase [61,62]. Ozkul et al. showed that LTG prevents increase in prefrontal cortex NO levels in rat global cerebral ischemia model [51]. Reactive oxygen species also disrupts cell structure and function by causing the production of MDA, a marker of lipid peroxidation, through the oxidation of polyunsaturated fatty acids in membrane phospholipids [63]. It has been suggested that in the experimental cerebral ischemia model, LTG can reduce oxidative-nitrosative stress by decreasing NO and MDA levels and increasing antioxidant GSH, GSH-R, SOD and CAT levels [51]. Previous studies had also shown that LTG had anti-epileptic activity in mice exposed to a chemoconvulsive model, and suggested that it may be associated with a decrease in oxidative stress with increase in CAT and GPx activities [64]. Moreover Tufan et al. [20], demonstrated that LTG improved the altered oxidant/antioxidant status by reducing the increased MDA levels and increasing the inadequate SOD and GPx levels, which formed due to traumatic spinal cord injury in rats. Consistent with previous studies, our results had shown that both LTG and MP treatment provided antioxidant and neuroprotective effects by increasing the SOD, CAT and GPx activities as well as decreasing MDA and NO levels after spinal cord ischemia in rats.

During the ischemia reperfusion excessive release of proinflammatory cytokines in addition to overproduction of ROS result in apoptotic cell death by activating the caspase cascade [65]. Caspase-3, as a reliable marker of apoptosis, initiates the final common pathway which causes break down proteins in the internal cytoskeleton and membrane of the cell [66-72]. Lagrue et al. [73], showed that, LTG prevented apoptosis by inactivation of the neuronal caspase 3 immunoreacivity in energy deficient mice. Kim et al. [74], further suggested that LTG exerts neuroprotective effect by suppressing cytochrome c release followed by activation of caspase-3. In this study, we demonstrated that caspase-3 activity as an indicator of apoptosis markedly elevated significantly after SCIRI. Both LTG and MP treatments protected spinal cord from apoptosis via inhibition of caspase-3 activity.

Ischemia-reperfusion injury caused neuronal pyknosis, intense axonal swelling, loss of cytoplasmic features and cytoplasmic eosinophilia at the cellular level. It also induced demolition of gray matter neurons. Additionally, SCIRI was shown to interrupt and separate the myelin configuration at the ultrastructural level especially in the large sized axons. These alterations were also partially improved by both LTG and MP treatments. Improvements in all these pathological changes also verified by light and electron microscopic examination.

Basso, Beattie and Bresnahan locomotor scale score and inclined-plane test were utilized for the assessment of locomotor recovery [33,34]. SCIRI leads to significant reduction in BBB scores as well as the mean angle in inclined-plane test, and these alterations have been substantially improved by both LTG and MP treatments when compared the ischemia group.

Although, safety of high-dose MP treatment in the acute spinal cord injury medication has been intensely questioned due to its side effects, it is still a well-known positive control group for SCIRI studies due to its antioxidant, antiinflammatory and antiapoptotic properties [10-15,30,75,76]. Therefore, we compared LTG treatment with the MP as a positive control group.

The insufficient number of rats in the groups, the short duration of the study, the lack of study of different drug administration routes, administration times and doses limit the adaptability of the results of our study to clinical use. The aim of administrating LTG immediately after reperfusion was to reach an effective blood concentration immediately after the insult. However, the effectiveness of the LTG aftrer SCIRI needs to be further be examined when applied after a certain period of time following the reperfusion, which would more likely mimic the actual clinical scenario. Therefore, based on these preliminary results, there is a need for more comprehensive, long-term studies involving different pathways in SCIRI.

Conclusion

Together with the results of previous studies, LTG may have beneficial effects in SCIRI by improving the biochemical, histological, ultrastructural and neurofunctional impairments toward enhancing neuronal survival via intraperitoneal administration of 20 mg/kg daily dose. In our opinion, these neuroprotective effects of LTG seems to be related its membrane stabilizing, antioxidative and antiinflammatory effects, which prevents the expansion of secondary excitotoxic neuronal damage.

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None.

Declarations of competing interest

None.

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