Original Article

Etanercept Prevents Histopathological Damage after Spinal Cord Injury in Rats

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Abstract

Background: The aim of our study is to assess the neuroprotective effects of the tumor necrosis factor alpha (TNF-α) inhibitor etanercept (ETA) on histopathological and biochemical changes following spinal cord injury (SCI). **Patients and Methods:** Fifty-four male Wistar albino rats were randomly assigned into three main groups: The sham, trauma, and ETA group (n = 18 per group). Each of these groups was further divided into three subgroups (n = 6 per subgroup) based on the different tissue sampling times postinjury: 1 h, 6 h, and 24 h. Clip compression model was used for SCI. Rats in the ETA group were treated with 5 mg/kg of ETA immediately after the clip was removed. After 1, 6, and 24 h, the spinal cord was totally removed between the levels T8–T10. Sample tissue was immediately harvested and fixed for histopathological and electron microscopic examination and were analyzed for TNF-α, interleukin-1β (IL-1β), superoxide dismutase (SOD), adenosine deaminase, catalase (CAT), and malondialdehyde levels in both the tissue and serum. **Results:** The serum and tissue levels of cytokines and enzymes were seen to change after SCI between hyperacute, acute, and subacute stages. Treatment with ETA selectively inhibited TNF-α, and IL-1β expression together with increased levels of antioxidative enzymes (SOD, CAT). **Conclusion:** Early administration of ETA after SCI may remarkably attenuate neuronal injury by decreasing tissue and serum TNF-α and IL-1β levels, while increasing antioxidative enzymes such as SOD and CAT in subacute and acute stages, respectively.

Keywords: *Etanercept, ischemia, neuroprotection, spinal cord injury*

Introduction

Neural damage occurs after spinal cord injury (SCI) as a result of oxidative stress, inflammation, and mitochondrial dysfunction.[1] Edema and hemorrhage may occur after the first 6–8 h of spinal trauma promoted by biochemical and inflammatory reactions.[2] These reactions promote both lipid peroxidation of the neural membrane and calcium ion influx into the cytoplasm, which leads to neuronal cell death and axonal sheath destruction.[3] Until recently, neuronal death caused by trauma was believed to be immediate and irreversible. However, neuronal damage after SCI is thought to continue for several days beyond the initial ischemic expansion that occurs within the first 24 h, a process called secondary damage. This second stage is characterized by a large number of cellular, molecular, and biochemical cascades with one of the most important mechanisms being the local inflammatory response.[4-6] The expression of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), regulates the precise cellular events occurring in the hours immediately after SCI and eventually the amount of secondary damage.

TNF-α is presumed to be involved in the local production of cytokines at the site of injury. Furthermore, this pro-death cytokine is involved in both necrosis and in the regulation of caspases along with other apoptotic factors.[7] Thus, it is not surprising that TNF-α is a member of the death-inducing ligand family, and triggers the extrinsic apoptotic pathway through its action on its two primary receptors, TNF receptor 1 (TNFR1) (p55), and TNFR2 (p75).

Etanercept (ETA) is a TNF-α inhibitor, such as adalimumab and infliximab, have been licensed for human therapies in a diverse set of peripheral inflammatory diseases such as rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, chronic asthma, and cholestasis.[8,9]

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Increased neuronal expression of cytokines TNF-α and interleukin-1β (IL-1β) occurs 1 h after acute SCI and reaches peak levels 4–24 h after SCI.[10-13] Understanding this pathophysiology offers the hope that better neuronal recovery can ultimately be achieved by intervening at this stage. Interestingly, recent evidence from rat models of stroke and mechanical spinal injury has suggested that anti-TNF-α strategies may be useful for the treatment of acute injury to the central nervous system.[5,14,15] Therefore, the goal of our current study was to assess the effects of ETA on the histopathological and biochemical changes following SCI.

Patients and Methods

Study design

Animal care and all experiments were in accordance with European Communities Council Directive of November 24, 1986 (86/609/EEC) on the protection of animals for experimental use. Furthermore, all procedures used in this investigation were reviewed and approved by the ethical committee of the Ankara Training and Research Hospital (Date: May 31, 2012; Number: 0009).

Fifty-four male Wistar albino rats weighing 300–350 g were randomly assigned to the following three main groups, which were further divided into subgroups as listed below:

- Sham group (n = 18): three subgroups of different time schedules. Rats underwent skin incision only, where nonischemic spinal cord samples were obtained 1 (n = 6), 6 (n = 6), or 24 h (n = 6) from postsurgery
- Trauma group (n = 18): three subgroups of different time schedules. Rats underwent SCI, and spinal cord samples were removed 1 (n = 6), 6 (n = 6), and 24 h (n = 6) immediately after trauma
- ETA group (n = 18): three subgroups of different time schedules Rats underwent SCI, and spinal cord samples were removed 1 (n = 6), 6 (n = 6), and 24 h (n = 6) immediately after trauma. All rats in this group received a single intravenous dose of 5 mg/kg of ETA instantly after the end of SCI.

Anesthesia and surgical procedure

Food and water were provided to the rats *ad libitum*. Anesthesia was performed with intramuscular administration of 40 mg/kg ketamine HCl (Ketalar®; Pfizer Inc., USA) and 5 mg/kg xylazine-HCl (Rompun® 2%; Bayer HealthCare AG, Germany). A dorsal laminectomy at thoracic level 8–10 (T8, T9, and T10) was performed, preserving the dura mater and exposing the dorsal (posterior) surface of the spinal cord. The aneurysm clip compression model of SCI used in the present study has been described previously by Rivlin and Tator.[16] Briefly, this model entails exposing the rat's spinal cord and applying an aneurysm clip with one blade under the ventral surface of the cord and the other over the dorsal surface. The clip (Yasargil, FE721, Aesculap, Germany), calibrated for a known compression force, was rapidly released and allowed to compress the cord for a predetermined amount of time before reopening and removal. After this procedure, all rats were allowed to recover from anesthesia, kept at normal room temperature and examined by a neurosurgeon to assess any development of neurological deficits. One, 6, and 24 h later, animals were resedated, and whole blood volumes removed through the abdominal aorta for biochemical examination. The dorsal incision made during the initial surgery was then reopened, and the spinal cord was totally removed between levels T8–T10. Sample tissues were immediately harvested and fixed for future biochemical, histopathological, and electron microscopic examination and divided into two equal parts with half of the spinal cord being stored at −30°C for biochemical examination, and the other half being stored at room temperature in 10% buffered formaldehyde solution for histological and electron microscopic examination.

Spinal cord tissue and serum biochemical analysis

Portions of spinal cord tissue were collected after SCI and were homogenized in phosphate-buffered saline containing 2 mM of phenylmethylsulfonylfluoride to evaluate the tissue levels of TNF-α, IL-1β, superoxide dismutase (SOD), adenosine deaminase (ADA), catalase (CAT), malondialdehyde (MDA), and glutathione peroxidase (GSH-Px). Blood serum levels of TNF-α, IL-1β, SOD, ADA, CAT, and MDA were also measured.

Histologic examination and analyses

Tissue processing for light microscopy

For histological examination, spinal cord tissue samples were fixed in 10% neutral-buffered formalin, dehydrated through a graded ethanol series and embedded in paraffin. Five-micron-thick paraffin sections were then obtained and stained with hematoxylin and eosin for analysis and photography by light microscopy (Leica DM 3000).

In all groups, histopathological changes due to acute injury were graded on a scale of 0–3 based on the following criteria.[17]

Grade 0 = No tissue damage.

Grade 1 = Mild neural tissue damage with rare necrotic neurons.

Grade 2 = Moderate neural tissue damage with necrotic neurons and white matter vacuolization.

Grade 3 = Severe neural tissue damage with widespread necrotic neurons and white matter hemorrhage, degeneration, and gliosis.

The same histologist, who did not know the identity of the groups, evaluated all animal specimens.

Tissue processing for electron microscopy

For electron microscopy, spinal cord specimens were fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate

buffer (pH 7.4) for 4–6 h at 4°C, postfixed in 1% osmium tetroxide for 2 h, dehydrated in graded ethanol solutions, and embedded in Araldite® (Huntsman, Istanbul, Turkey). Semi-thin sections (1 μm) were then obtained and stained with toluidine blue for subsequent light microphotography and observation. Ultra-thin sections were also obtained and stained with uranyl acetate plus lead citrate for observation under an LEO 906 E transmission electron microscope.

Statistical analyses

Data were analyzed using the Statistical Package for Social Sciences software version 19.0 for Windows (SPSS Inc., Chicago, IL, USA), and nonparametric tests were applied. The Mann–Whitney U-test was used to compare two independent groups, while the Kruskal–Wallis test was used to compare more than two groups. The Wilcoxon signed-rank test was used to compare two dependent groups, while the Friedman test was used to compare more than two groups. Bonferroni's correction for multiple tests was used for *post hoc* comparisons. All differences associated with a chance probability of 0.05 or less were considered statistically significant. Continuous variables are presented as mean ± standard deviation.

Results

It was determined that spinal tissue levels of IL-1β in the ETA group were significantly lower than that of the trauma groups during the hyperacute (1 h post-SCI) and acute (6 h post-SCI) stages postinjury. In addition, spinal tissue CAT levels in the ETA group were significantly higher than that of the trauma group in the acute stage. The spinal tissue levels of the MDA and GSH-Px levels were comparable among sham, trauma, and ETA groups in the hyperacute, acute, or subacute (24 h post-SCI) stages. Furthermore, spinal tissue levels of SOD and ADA were comparable among sham, trauma, and ETA groups in the hyperacute, acute, or subacute (24 h post-SCI) stages. However, spinal tissue levels of TNF-α and MDA during the subacute stage were significantly higher than those of the acute and hyperacute stage in the ETA group, whereas CAT levels in the subacute stage were significantly lower than that of the hyperacute stage in the ETA group [Figures 1 and 2].

It was determined that serum levels of IL-1β in the ETA group were significantly lower than that of the trauma group in the hyperacute and subacute stages [Figure 3]. In addition, serum SOD levels in the ETA group were significantly lower than that of the trauma group in the hyperacute and acute stages but were significantly higher than that of the trauma group in the subacute stage. Furthermore, serum SOD levels in the subacute stage were significantly higher than that of the hyperacute stage in the ETA group. Serum ADA levels in the ETA group were significantly lower than that of the trauma group at the hyperacute and acute stages, and serum MDA levels of the ETA group were significantly lower than that of the trauma group at the hyperacute stage [Figure 3].

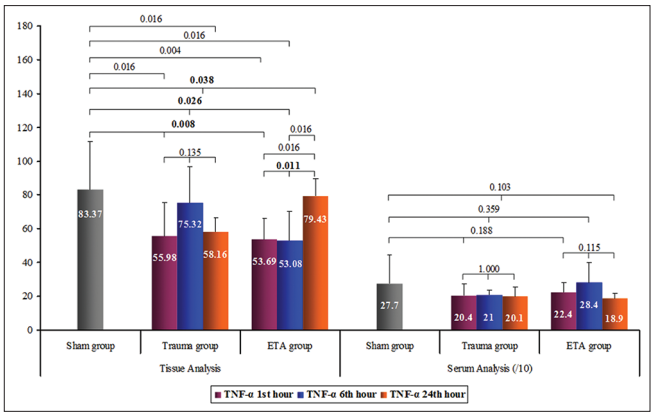

Figure 1: Biochemical analysis of tumor necrosis factor-α in tissue and serum in sham, trauma, and etanercept groups. Data are shown as median ± interquartile range

Not surprisingly, all tissue obtained from rats in the sham group scored a Grade 0 in that there was no nerve tissue damage in the spinal cord. Furthermore, neurons and neuroglia cells were normal in appearance in both white and gray matter.

With the histopathological evaluation of spinal cord trauma and ETA applied after trauma in respect of the 1, 6, and 24 h groups, the semi-thin sections showed tissue damage and areas of hemorrhage in both gray and white matter [Figures 4a-c,e, 5a, and 6c]. Blood vessels showed perivascular erythrocyte extravasation [Figure 4d]. Degenerating neurons showing prominent central chromatolysis and pyknotic nuclei were observed [Figures 4e, f and 5e, f]. Neuropil, the fibrillary matrix formed by the cellular extensions of the neurons and glial cells, showed vacuolization and degenerative changes [Figures 4b, c, e, and 5c, d, f]. We also observed swollen astrocytic extensions forming clear spaces around injured neurons and blood vessels [Figures 4a, b and 5b, d, f].

Ultrastructural examination of the 1, 6, and 24 h, ETA-applied posttrauma groups' ultra-thin sections showed degenerating neurons with swollen mitochondria, distended cisternae of the rough endoplasmic reticulum, and highly electron dense cytoplasm [Figures 6a and 7e]. In the 6 h trauma group, neurons showed chromatin clumping in the nucleus demonstrating karyorrhexis [Figure 6d]. Surrounding neuropil includes erythrocytes due to hemorrhage and characterized by swollen processes of neuronal and glial cells [Figures 6a-f and 7a-f]. Myelinated fibers exhibited disorganization of the myelin periodic pattern and rupture of the myelin sheath. Some degenerated axons with swollen mitochondria in altered axoplasm were observed [Figures 6b, c, e, f and 7a-f].

The spinal cord tissue samples obtained from trauma group animals after 1 and 6 h of SCI was scored

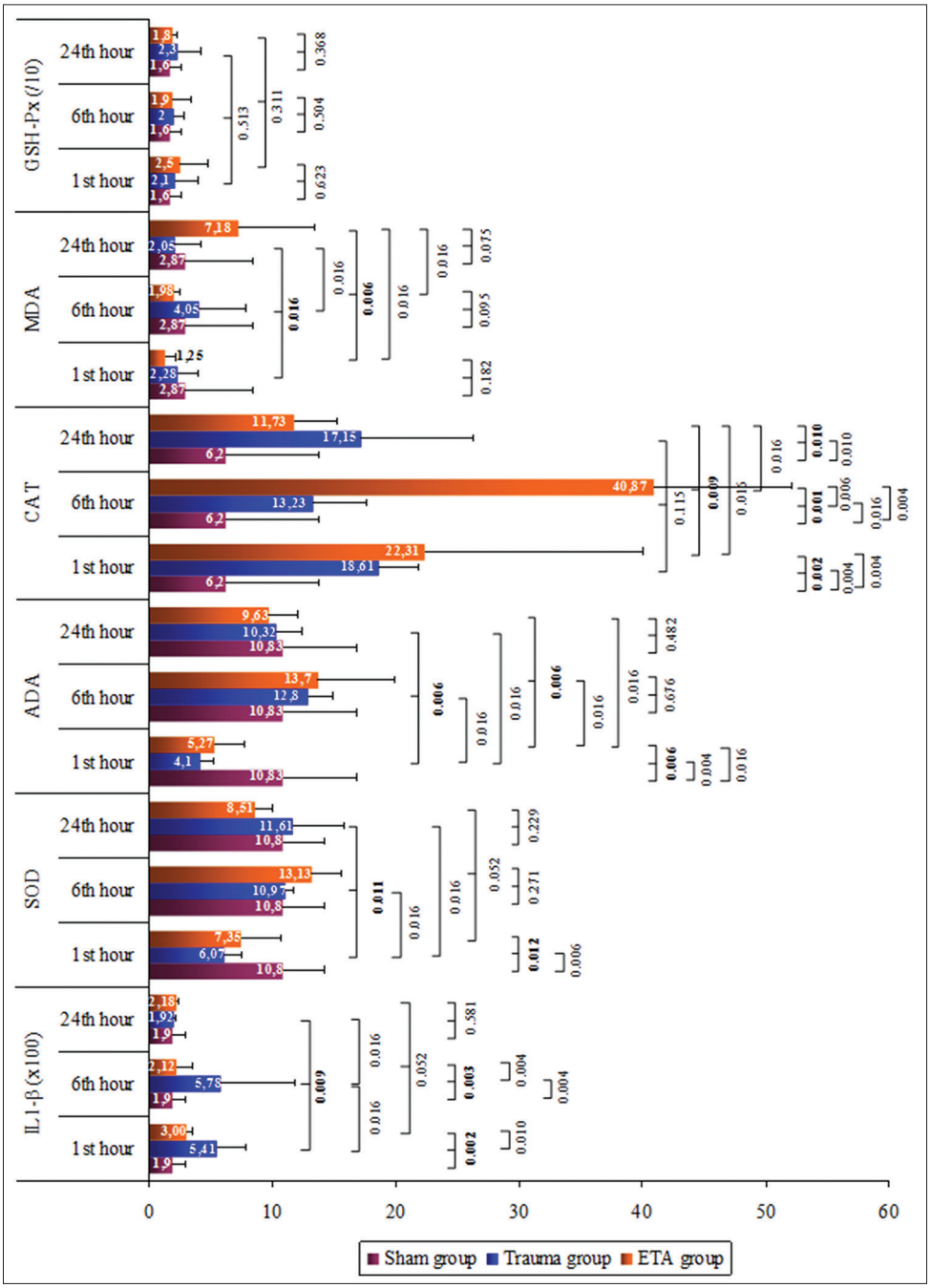

Figure 2: Biochemical tissue analysis in sham, trauma, and etanercept groups (median ± interquartile range)

Table 1: Histopathological grade distribution among groups after spinal cord injury

Median (minimum-maximum)						
Sham	**Trauma-1**	**Trauma-6**	**Trauma-24**	**ETA-1**	**ETA-6**	**ETA-24**
0 (0-0)	*2 (1-3)	*2.5 (2-3)	*3 (2-3)	1 (1-2)	1 (1-1)	**2 (2-3)

*Between trauma and sham groups $P<0.05$, **Between ETA-6 and ETA-24 h groups $P<0.05$. ETA – Etanercept

as Grade 2, whereas spinal cord tissue samples obtained after 24 h of SCI was scored as Grade 3. There are statistically significant differences between histopathological grading of sham and trauma groups [$P < 0.05$; Table 1].

Although the histopathological grades of the ETA group were improved in comparison with the trauma alone groups, the histopathological grades of the ETA group were higher at the 24 h postinjury time point, and this difference was statistically significant when compared to the ETA

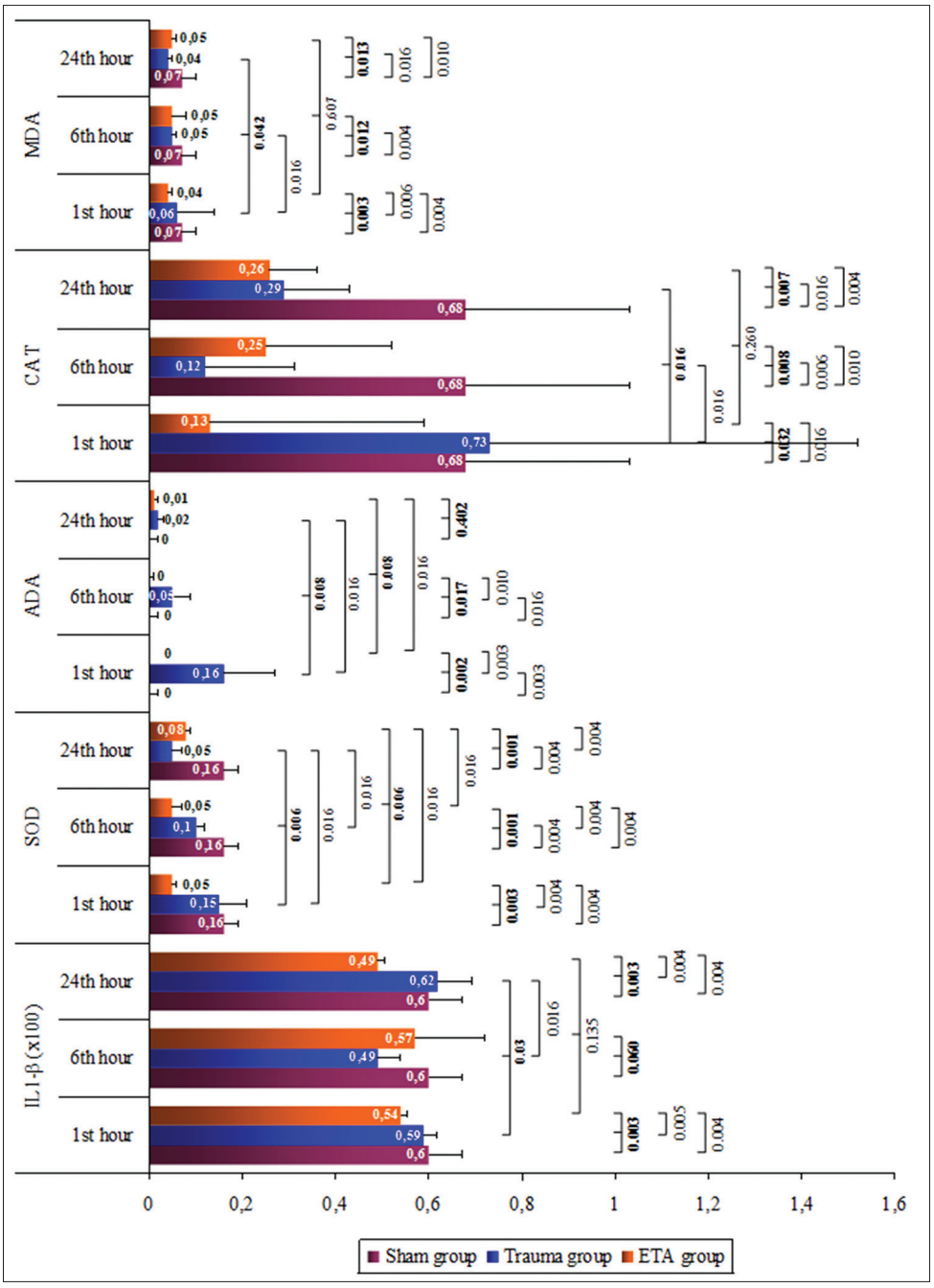

Figure 3: Biochemical serum analysis in sham, trauma, and etanercept groups (median ± interquartile range)

6-h group [$P < 0.05$; Table 1]. Moreover, in hematoxylin and eosin stained sections, eosinophilic neurons indicating damage were observed to be more numerous in the 24-h ETA group than in the 1- and 6-h ETA groups.

Discussion

In this study, the protective effect of ETA in the SCI model was evaluated with the help of the measurement of cytokines in spinal cord tissue and serum levels at the hyperacute (1 h post-SCI), acute (6 h post-SCI), and subacute (24 h post-SCI) period after the injury. The histopathology of the spinal cord tissue was also evaluated with by light microscopy and electron microscopy within the same period. We found that a single intravenous dose of ETA (5 mg/kg) administered immediately after spinal trauma reduced the IL-1β level in the hyperacute (1 h) and acute (6 h) stages in the spinal cord tissue samples and in the hyperacute and subacute stages in the serum samples. The ETA also suppressed the TNF-α level in the spinal cord tissue samples at the hyperacute and acute stages after spinal trauma. During the period after ETA administration, histopathological evaluation of the damaged spinal cord tissue sample revealed a better light microscopic and electron microscopic finding, especially

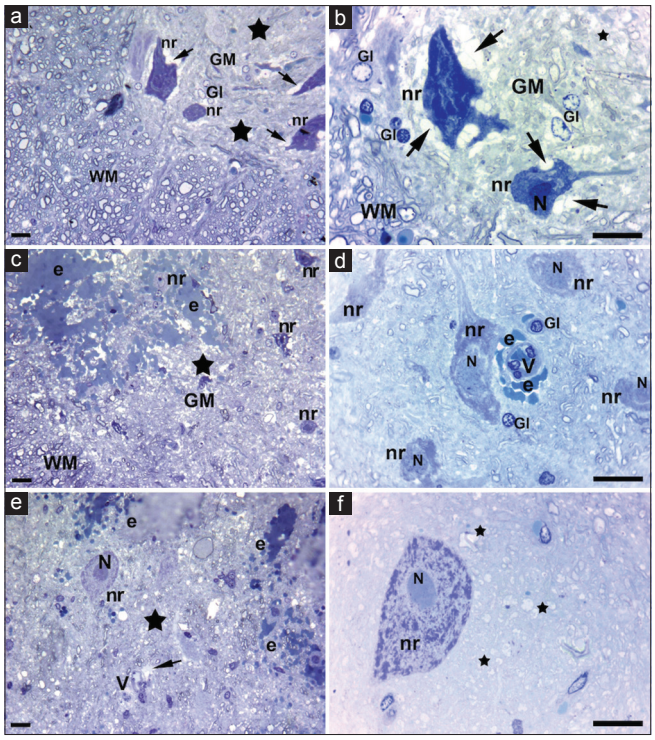

Figure 4: Spinal cord tissue semi-thin sections of postinjury groups ([a and b] 1 h, [c and d] 6 h, [e and f] 24 h). (a-d) GM: Gray matter, WM: White matter, nr: Neuron, Gl: Glial cell, stars: Neuropil, V: Blood vessel, e: Erythrocyte, Arrows: Swollen astrocytic processes around neuron, N: Nucleus, (e-f) N: Pyknotic nucleus and central chromatolysis in neurons. Epon embedded toluidine blue, Bars: 20 µm

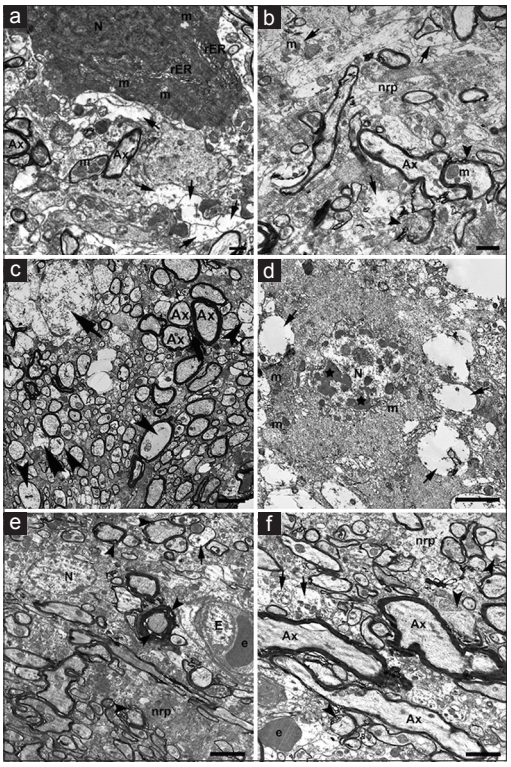

Figure 5: Spinal cord tissue ultra-thin sections of postinjury groups ([a and b] 1 h, [c and d] 6 h, [e and f] 24 h). (a) N: Nucleus of neuron, m: Swollen mitochondria, rER: Distended cisternae of rough endoplasmic reticulum, Ax: Myelinated axon, Arrows: Swollen astrocytic processes, (b and c) nrp: Neuropil, Arrowhead: Rupture of the myelin sheath and degenerated axoplasm, (d) m: Mitochondria, N: Karyorrhexis, stars: Chromatin clumping, Arrows: Swollen cell processes, (e and f) e: Erythrocyte, E: Endothelium, nrp: Neuropil, N: Nucleus of a glial cell, Ax: Axoplasm, Arrowhead: Myelin sheath disorganization and rupture, Arrows: Swollen cell processes. (a and b): ×6000, Bars: 1000 nm; (c): ×2784, Bar: 2500 nm; (d): ×4646, Bar: 2500 nm; (e and f) ×3597, Bars: 2500 nm, transmission electron micrograph

in the hyperacute and acute stages after spinal trauma. The present study provides evidence of the neuroprotective effect of intraperitoneally administered ETA posttreatment in a rat model of SCI. Beneficial effects were observed both histologically and biochemically.

Injury to the spinal cord causes primary and secondary injury processes. Primary injury is related to direct damage to nerve cells, axons, and vascular structures leading to hemorrhage, edema, lysosomal depletion, demyelination, cystic necrosis, and ischemia. In contrast, secondary mechanisms of injury occur primarily at the cellular level and involve delayed effects of ischemia, edema, excitotoxicity, mitochondrial dysfunction, low cellular energy status, and the induction of free radical-induced lipid peroxidation, which has often been proposed as an important factor in posttraumatic neuronal degeneration.[1] More specifically, traumatic SCI results in enhanced generation of superoxide anions or their conversion to other free radicals which can destroy membrane integrity by lipid peroxidation, known as oxidative injury.[8,17-19] This pro-inflammatory response is biphasic with the first phase involving lysosomal degradation and free radical formation, which play a role in the chemotaxis of neutrophils and tissue macrophages. The second phase includes phagocytosis of the damaged neural tissue by macrophages and histiocytes.[20] Several clinical studies have suggested that certain pharmacological therapies are effective in minimizing the damage incurred after SCI. It has been underlined that pharmacological agents used for ischemia related to surgical clamp and following reperfusion injury in thoracic and thoracoabdominal surgeries minimize SCI.[21] MDA is a lipid peroxidation product which increases immediately after SCI.[22] ADA is an important enzyme participating in purine and DNA metabolism, and it converts adenosine to inosine, which plays a neuroprotective role after ischemia.[23-25] SOD, CAT, and GSH-Px are antioxidative enzymes which have beneficial effects on the oxidative injury.[23,26,27] Besides its reducing effects on oxidative stress and increasing effects on the antioxidative properties of N-acetylcysteine, it has also been proved to make clinical and histopathological improvements in experimental SCI models.[28]

In this study, spinal cord tissue CAT of the ETA group was significantly higher than that of the trauma group in the acute stage. Spinal cord tissue MDA and tissue GSH-Px levels in the ETA group were not significantly different from those of the trauma or sham groups in hyperacute,

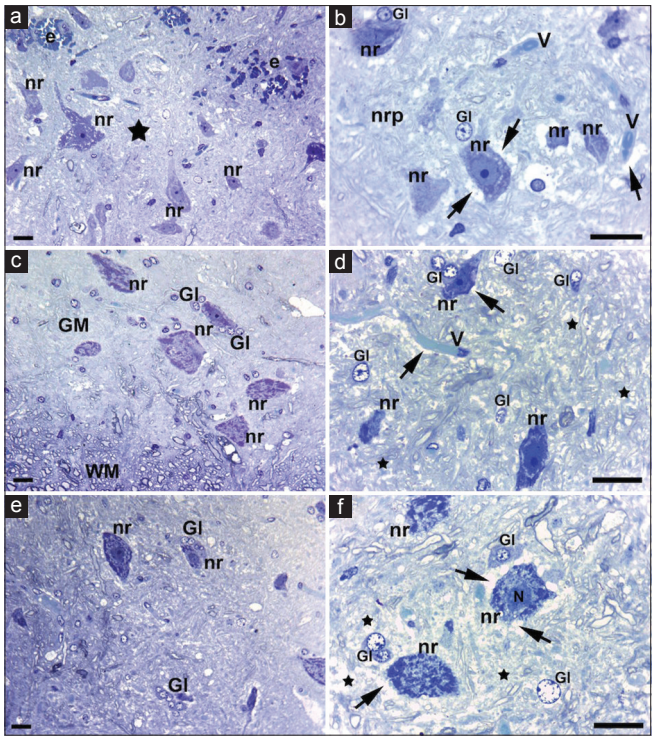

Figure 6: Spinal cord tissue semi-thin sections of etanercept-treated groups ([a and b] 1 h, [c and d] 6 h, [e and f] 24 h). (a and b) nr: Neuron, Gl: glial cell, Stars: Neuropil, e: Erythrocyte in hemorrhage areas, Arrows: Swollen astrocytic processes around neuron, V: Blood vessel, (c and d) GM: Gray matter, WM: White matter, Arrows: Swollen astrocytic processes around neuron and blood vessel, (e and f) N: Pyknotic nucleus and central chromatolysis in neurons. Epon embedded toluidine blue, Bars: 20 μm

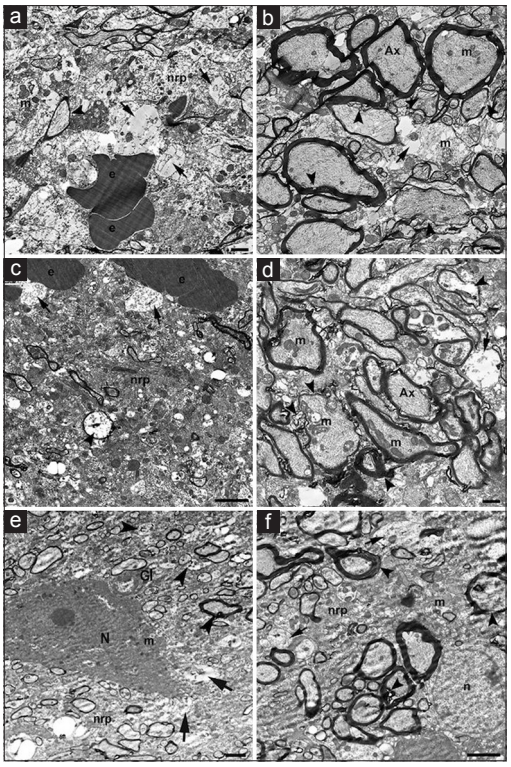

Figure 7: Spinal cord tissue ultra-thin sections of Etanercept-treated groups ([a and b] 1 h, [c and d] 6 h, [e and f] 24 h). (a and b) Nrp: Neuropil, m: Swollen mitochondria, Arrows: Swollen cell processes, Arrowhead: Thin myelin sheath and disorganization of myelin lamellae, e: Erithrocyte in parenchyma, Ax: Axoplasm, (c and d) nrp: Neuropil, Arrowhead: Degenerative changes in myelin sheath, m: Mitochondrion in axoplasm, Arrow: Swollen and degenerated cell processes, e: Erythrocyte in haemorrhage areas, (e and f) nrp: Neuropil, N: Nucleus, m: Mitochondrion of a neuronal cell, Gl: Glial cell, Arrowhead: Myelin sheath disorganization, Arrows: Cell processes, n: Nucleus of a glial cell. (a, b and d): ×6000, Bars: 1000 nm; (c and f): ×4646, Bar: 2500 nm; (e): ×2784, Bar: 2500 nm, transmission electron micrograph

acute, and subacute stages following SCI. Further, tissue TNF-α, SOD, and ADA levels in all postinjury stages were not significantly different from each other among any of the groups. Moreover, serum SOD levels in the ETA group were significantly lower than that of the trauma group in the hyperacute and acute stages, while significantly higher than that of the trauma group in the subacute stage. Total amounts of ADA in the serum of the ETA group were significantly lower than that of the trauma group in both hyperacute and acute stages of post-SCI, and MDA serum levels in the ETA group were significantly lower than that of the trauma group in the hyperacute stage following injury.

On histopathological examination, within the hyperacute stage of the trauma group one rat exhibited Grade 3 degeneration while no rats displayed Grade 3 degeneration in the hyperacute stage of the ETA group. In the acute stage, three rats had Grade 3 degeneration in the trauma group, while all rats displayed Grade 1 degeneration in the ETA group. Finally, in the subacute stage, all rats in the trauma group exhibited Grade 3 degeneration, while only two rats had Grade 3 degeneration in the ETA group.

These histopathological results suggest that ETA can play a role in rescuing tissue from damage due to SCI, but that a single dose may be insufficient to maintain its beneficial effects through 24 h postinjury.

Although the precise mechanisms underlying SCI remain unclear, there is evidence that early inflammatory events promote tissue damage in the acutely injured spinal cord.[29] An inflammatory response develops within hours after injury and is characterized by infiltration of neutrophils and activation of microglia.[30] This is followed by a second response aimed to localize the inflammatory reaction within the spinal cord tissue. Moreover, it has been well-demonstrated that the local expression of pro-inflammatory cytokines following SCI, including TNF-α and IL-1β, regulates the precise cellular events that occur after SCI.[31]

Several studies have reported that TNF-α is involved in the secondary injury that occurs after SCI, suggesting that this cytokine could serve as a potential target of therapeutic inhibition. TNF-α and IL-1β, pro-inflammatory cytokines, play an important role in the production of nitric oxide (NO) by the induction of nitric oxide synthase (iNOS).[32] NO is a highly reactive free radical and high levels of NO are known to be neurotoxic. Moreover, iNOS has been linked to secondary damage,

which ultimately leads to apoptotic cell death.[32,33] Injection of IL-10, a potent anti-inflammatory cytokine, has been reported to reduce TNF-α production in the spinal cord and improve functional recovery after SCI.[34]

ETA reduced the cytokine expressions of both TNF-α and IL-1β, which have been proven to be beneficial in an animal model of SCI.[5] More specifically, when ETA (5 mg/kg) was given 1 h before SCI, it reduced the activity of TNF-α and IL-1β which decreased the degree of spinal cord inflammation, iNOS, apoptosis, and tissue injury. Consequently, pretreatment with ETA improved the functional prognosis of the animal in the experimental SCI.[5] Immediate inhibition of TNF-α by ETA administration after the nerve injury enhanced the rate of axonal regeneration in rats.[35] In addition, TNF-α has been shown to inhibit neurite outgrowth in cultured dorsal root ganglia and hippocampal neurons.[36,37]

In the present study, tissue IL-1β levels in the ETA group were significantly lower than that of the trauma group, both in the hyperacute and acute stages following SCI. We also observed that serum levels of IL-1β in the ETA group were significantly lower than that of the trauma group in both the hyperacute and subacute stages following injury. The TNF-α levels of the ETA group were significantly suppressed in hyperacute and acute stages when compared with the subacute stage in the spinal cord tissue samples after SCI. It was shown that the administration of ETA decreased IL-1β levels both in the spinal cord tissue and serum samples after SCI within the first 24 h. ETA also depressed the TNF-α level in spinal cord tissue after SCI at the hyperacute and acute stages. The introduction of ETA increased SOD and CAT levels in both the subacute and acute stages. These findings indicated that ETA had blocked the TNF-α and IL-1β and increased the levels of antioxidative enzymes such as CAT and SOD, which may have a beneficial effect in the early period after experimental SCI. These results were supported with the histopathological observation in this study. These previous reports, along with our current findings, provide strong support for ETA as a therapeutic agent when it is administered early after trauma.

Conclusion

ETA decreases TNF-α and IL-1β levels while increasing antioxidative enzymes such as SOD and CAT in both the subacute and acute stages. Therefore, early administration of ETA may ameliorate histopathological damage occurring after SCI.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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