● RESEARCH ARTICLE

Topiramate as a neuroprotective agent in a rat model of spinal cord injury

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Abstract

Topiramate (TPM) is a widely used antiepileptic and antimigraine agent which has been shown to exert neuroprotective effects in various experimental traumatic brain injury and stroke models. However, its utility in spinal cord injury has not been studied extensively. Thus, we evaluated effects of TPM on secondary cellular injury mechanisms in an experimental rat model of traumatic spinal cord injury (SCI). After rat models of thoracic contusive SCI were established by free weight-drop method, TPM (40 mg/kg) was given at 12-hour intervals for four times orally. Post TPM treatment, malondialdehyde and protein carbonyl levels were significantly reduced and reduced glutathione levels were increased, while immunoreactivity for endothelial nitric oxide synthase, inducible nitric oxide synthase, and apoptotic peptidase activating factor 1 was diminished in SCI rats. In addition, TPM treatment improved the functional recovery of SCI rats. This study suggests that administration of TPM exerts neuroprotective effects on SCI.

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Introduction

Spinal cord injury (SCI), complete or partial, is a significant public health problem because of its consequences due to loss of motor, sensory, autonomic, and reflex functions of the spinal cord. SCI is thought to occur *via* two mechanisms: a primary mechanical injury and a secondary injury in which one or multiple mechanisms, triggered by the primary injury, are involved (Azbill et al., 1997; Dumont et al., 2001). Currently, there are no treatment options, except prevention, to manage destruction and primary injury following SCI. Therefore, most experimental and clinical studies on SCIs aim to prevent secondary injury. Major mechanisms mediating secondary injury include, but are not limited to, ischemia, glutamate excitotoxicity, inflammation, free oxygen radicals, apoptosis, cytoskeletal degradation, and demyelination (Azbill et al., 1997; Lu et al., 2000; Dumont et al., 2001; Park et al., 2004). Notably, following spinal cord trauma, excessive amounts of glutamate are released, and neural and glial cells are lost via excitotoxic and other secondary mechanisms (Wrathall et al., 1996; Park et al., 2004).

Topiramate (TPM) is an antiepileptic drug that has a wide variety of applications in clinical practice (Dinoff et al., 2003; Shank and Maryanoff, 2008). TPM inhibits voltage-gated Na+ channels and selectively antagonizes glutamate receptor subtypes kainate and α-amino-3-hydroxy-5-methyl isoxazole propionate (AMPA) receptors (Dinoff et al., 2003; Gensel et al., 2013). Additionally, it weakly inhibits the carbonic anhydrase (CA) (Dodgson et al., 2000). Its beneficial effects in neurological recovery have been demonstrated, albeit without significant anatomical/histopathological changes, in two experimental models of traumatic brain injury (TBI) (Hoover et al., 2004; Kouzounias et al., 2011). In some experimental stroke studies, TPM decreased the infarct volume and showed antioxidant effects. Moreover, it is considered to be protective against neurological damage following severe ischemia/hypoxia in neonates (Shank et al., 2000). Although TPM is well-tolerated and beneficial in reducing post-injury pain in patients with SCI (Dinoff et al., 2003), its use in SCI has not been extensively studied. Only one study in the literature reported neuroprotective and oligodendroglia-sparing effects of TPM in a model of unilateral cervical SCI (Gensel et al., 2013). In the present study, we aimed to investigate antioxidant, antiapoptotic, neuroprotective, and behavioral effects of TPM in an experimental rat model of thoracic contusive SCI.

Materials and Methods

Animals

Experimental protocols performed in this study were approved by Hacettepe University Animal Research Ethics Committee with the approval number 2009/15-2 and were in compliance with the European Union (EU) directive (2010/63/EU) on the protection of animals used for scientific purposes. Forty 6–8-week-old male Wistar rats weighing 150–200g were used. Animals were housed in Hacettepe University Experimental Animal Facility at room temperature under a 12-hour dark/light cycle and permitted food and water *ad libitum*.

The rats were randomly divided into four groups with 10 rats in each group. Rats in sham-operated group underwent only laminectomy. Rats in SCI only group underwent laminectomy and SCI induction but did not receive any treatment. Rats in the TPM group were administered 40 mg/kg TPM (TOPAMAX®, Janssen Pharmaceuticals, Raritan, NJ, USA) (2 mL, dissolved

in saline) intraperitoneally 30 minutes after laminectomy and SCI induction and via oral gavage (2 mL, dissolved in saline) at 12, 24, 36, and 48 hours after the procedure. The TPM dosage regimen was selected based on previous studies with an experimental rat model of TBI (Hoover et al., 2004; Kouzounias et al., 2011). Rats in the SCI + vehicle group were administered equivalent volumes (2 mL) of 0.9% isotonic saline solution intraperitoneally 30 minutes after injury and *via* oral gavage at 12, 24, 36, and 48 hours after injury.

Furthermore, five rats in each group were randomly selected and sacrificed 24 hours after SCI (the remaining five rats in each group were followed for 4 weeks before sacrifice). Under general anesthesia by intraperitoneal injection of 60 mg/kg ketamine hydrochloride (Ketalar 5% solution, Eczacıbaşıİlaç Sanayi under Parke-Davis license; Istanbul, Turkey) and 8 mg/kg xylazine (Rompun 2% solution; Bayer, Istanbul, Turkey), rats underwent intracardiac perfusion of saline. Additionally, 1-cm-long spinal cord segments encompassing the injury site (approximately the T_9 level) were harvested under microscopic guidance. These segments were divided into two pieces (at the level of the trauma epicenter) for biochemical and immunohistochemical studies. The remaining rats were followed up for 4 weeks and were evaluated for their behavioral/functional impairment using motor function scores (MFS) and inclined plane scores (IPS) at the end of that period. Following functional evaluation, those rats (*n* = 5 rats/group) were similarly sacrificed and1-cm-long spinal cord segments encompassing the injury site (approximately the T_9 level) were harvested under microscopic guidance and embedded in paraffin to be used for histopathological analysis and lesion area measurements.

Surgical procedure
All animals were anesthetized by intraperitoneal injection of 60 mg/kg ketamine hydrochloride and 8 mg/kg xylazine. Following anesthesia, all rats were placed in the prone position and skin incision and paravertebral muscle dissection were performed at the T_{5-12} and T_{7-11} vertebral levels, respectively. The dura was exposed following microscope-assisted laminectomy at the T_{8-10} vertebral levels.

A weight-drop model, modified from Allen's method (Koozekanani et al., 1976), was used to induce traumatic SCI through a custom-made device. Force was applied by dropping a 5 g weight of spherical lead through a 10-cm guide tube, which was positioned perpendicular to the exposed spinal cord. Immediately after trauma, the muscles and skin incision were sutured using 3-0 silk sutures. The rats were returned to a temperature- and humidity-controlled chamber. Manual expression was performed thrice per day, until reflex emptying of the urinary bladder was observed.

Histopathological evaluation using hematoxylin and eosin
At the end of 4 weeks, five rats from each group were deeply anesthetized and 4% paraformaldehyde was perfused intracardially. Spinal cord segments around the injury site (T9 level) were removed under the microscope (Zeiss OPMI99, Oberkochen, Germany) and left in 10% formaldehyde for 1 week. For pathological evaluation, sections were cut (5 μm

thick) from formalin-fixed and paraffin-embedded spinal cord segments. They were stained with hematoxylin and eosin. The slides were viewed under light microscopy to examine structural changes. Lesion size and total spinal cord cross-sectional area were measured at the lesion epicenter level. The proportion of the lesion size to the total area of spinal cord on each section was recorded as a percentage. Three sections with the largest lesion area were evaluated and percentages were averaged for each animal.

Biochemical analysis
For biochemical analyses, five rats from each group were sacrificed 24 hours after SCI. The harvested samples were stored at −20°C for further biochemical analysis, *i.e.*, lipid peroxidation (MDA), and determination of protein carbonyl (PC) and reduced glutathione (GSH) levels.

To analyze lipid peroxidation, malondialdehyde (MDA) level was measured, as described previously (Miharaand Uchiyama, 1978). After weight measurement, frozen tissue samples were homogenized in 1:10 (w:v) potassium phosphate buffer (50 mM, pH 7.4) using a Dounce homogenizer. The homogenate (0.5 mL) was mixed with 3 mL 1% phosphoric acid and 1 mL 0.67% thiobarbituric acid (TBA) was added. Tubes were placed in boiling water for 45 minutes. After cooling the tubes, TBA-reactive substances (TBARS) were extracted into n-butanol and the absorbance was measured at 532 nm. The molar absorptivity of the TBA–MDA complex was taken as 1.56×10^5/M/cm; thus, tissue lipid peroxide levels (as TBARS) were calculated as nanomole per gram wet tissue (nmol/g).

To evaluate the oxidative damage, the tissue PC level was measured, as described previously (Levine et al., 1990) using assay kits (Cayman Chemical Company, Ann Arbor, MI, USA). Proteins were precipitated by adding 20% trichloroacetic acid and were redissolved in dinitrophenylhydrazine, and the absorbance was read at 370 nm. The PC level was expressed as nanomole per milligram tissue (nmol/mg).

Reduced glutathione (GSH) levels were calculated using assay kits (Cayman Chemical Company) by the Ellman method (Ellman, 1959). GSH is reacted with 5,5′-dithiobis (2-nitrobenzoic acid) resulting in the formation of a product that has absorbance at 410 nm. Results were expressed as nanomole per gram tissue (nmol/g).

Immunohistochemical evaluation
Five rats in each group were sacrificed 24 hours after SCI, and spinal cord segments at the T_9 level and below were harvested. Sections were cut (5 μm thick) from formalin-fixed and paraffin-embedded spinal cord segments and were prepared for immunohistochemical staining. Moreover, a few slices were stained with hematoxylin and eosin and were evaluated to confirm SCI (**Figure 1A**). All sections were applied to poly-L-lysine-coated slides and were dewaxed in xylene. Sections were washed in decreasing series of ethanol and water. Cold hydrogen peroxide (3%) in distilled water was applied to block endogenous peroxidases for 30 minutes. Furthermore, the sections were washed with phosphate buffered saline (PBS) and incubated at 4°C with an endothelial nitric oxide synthase (eNOS) rabbit polyclonal antibody (Lab Vision Corp.,
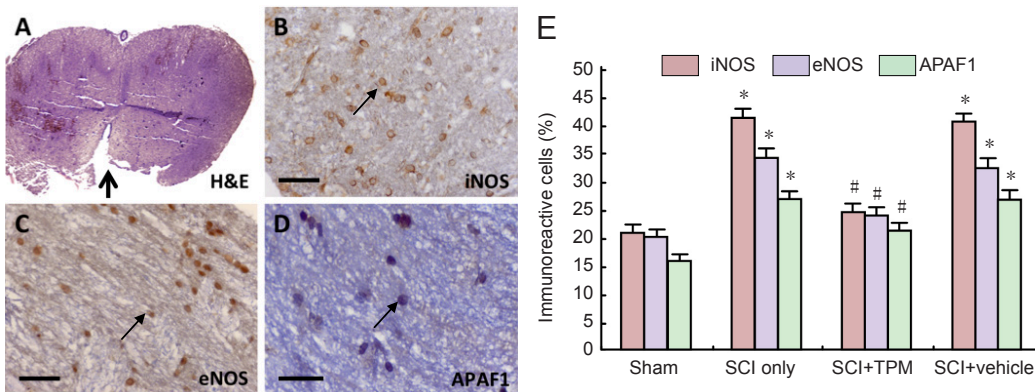
Figure 1 Representative microscopic images of spinal cord sections (A–D) and quantification of iNOS, eNOS, and APAF-1 immunoreactivity (E) in experimental groups at 24 hours following SCI.
(A) Hematoxylin and eosin (H&E)-stained sections showing injury on the dorsal part (arrow). (B–D) Immunohistochemical staining for iNOS, eNOS, and APAF-1 in spinal cord sections taken 24 hours after injury (original magnification, 400×). Arrows show iNOS-, eNOS-, and APAF-1-immunoreactive cells. Scale bars represent 50 μm. (E) The graph shows iNOS, eNOS, and APAF-1 immunoreactivity (% cells). Results were expressed as the mean ± SE. *$P = 0.001$, *vs.* sham(-operated) group; #$P = 0.01$, *vs.* SCI only and SCI + vehicle groups (Mann-Whitney U test; $n = 5$ rats/group. iNOS: Inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase; APAF-1: apoptotic peptidase activating factor 1; SCI: spinal cord injury; TPM: topiramate.

Table 1 Motor function score (MFS) proposed by Farooque et al. (1999)

Score 0	No movement of the hindlimbs
Score 1	Barely perceptible movement in hindlimbs
Score 2	Brisk movements at most hindlimb joints (hip, knee or ankle) in one or both limbs but no coordination, no weight support
Score 3	Alternate stepping and propulsive movements of hindlimbs, no weight support
Score 4	Can support weight on hindlimbs
Score 5	Walk with only mild deficit
Score 6	Normal walking

Fremont, CA, USA), inducible NOS (iNOS) rabbit polyclonal antibody (Lab Vision Corp., Fremont, CA, USA), or apoptotic peptidase activating factor 1 (APAF-1) epitope specific rabbit antibody (Lab Vision Corp., Fremont, CA, USA), at a dilution of 1/200 in PBS for NOS antibody and at 1/100 for APAF-1 antibody, for 16 hours at 4°C. Moreover, the sections were incubated with biotinylated goat anti-rabbit IgG secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA), washed with PBS thrice, and incubated with an avidin-biotin-peroxidase complex and were then visualized using a chromogenic reaction with diaminobenzidine (DAB). Slides were examined under the microscope (Leica, DM2000) by an experienced histopathologist who was blinded to the study groups (**Figure 1B–D**). For each animal, three sections around the lesion epicenter were evaluated. For each section, five random regions (including gray and white matter) were inspected with 40× magnification. The percentage of positively immunolabeled cells over total cells in each region was determined. Then, average percentage for five regions in each section was calculated. This calculation was performed for three tissue sections per animal, and the overall average percentage was calculated for each animal.

Behavioral evaluation
We assessed behavioral changes in rats with the hind limb motor function score (MFS) and inclined plane score (IPS) at 4 weeks post-injury. MFS was measured, as described previously (Farooque et al., 1999). A description of MFS is given in **Table 1**. The rats were observed for 1 minute while moving on a horizontal plane of 0.7×0.9 m². The hip, knee, and ankle joint movements were recorded.

We also used IPS (Rivlin and Tator, 1977) to evaluate behavioral changes in the rats. The highest angle at which a rat can support its weight for 5 seconds on an inclined plane (30×60 cm²) measured at 0°–90° was recorded. MFS and IPS were evaluated by a neurosurgeon blinded to the study groups and results were compared between groups.

Statistical analysis
Data were analyzed using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). Results were expressed as the mean ± SE. For statistical comparisons between groups, the Kruskal-Wallis test was used, and for dual comparison, the Mann-Whitney U test was used. A P value of < 0.05 was considered statistically significant.

Results
Oxidative stress
A comparison of biochemical findings among all experimental groups at 24 hours after SCI is illustrated in **Figure 2**. MDA levels were significantly increased in the SCI only and SCI + vehicle groups than in the sham-operated group (all $P < 0.01$). MDA levels in the SCI + TPM group were significantly reduced compared with those in the SCI + vehicle group ($P = 0.005$).

In the SCI + TPM group, MDA levels were significantly reduced compared with those in the SCI + vehicle group ($P = 0.005$). Nevertheless, there was still statistically significant difference between sham-operated and SCI + TPM groups ($P < 0.001$).

In the SCI and SCI + vehicle groups, PC levels were significantly increased compared with those in the sham-operated group ($P < 0.001$). PC levels were significantly reduced in the

SCI + TPM group than in the SCI + vehicle group (P = 0.04). Despite this reduction, there was still a statistically significant difference between sham-operated and SCI + TPM groups (P = 0.01). Reduced GSH levels were significantly diminished in the SCI only and SCI + vehicle groups than in the sham-operated group (P < 0.001). Reduced GSH levels in the SCI + TPM group were significantly elevated compared with those in the SCI + vehicle group (P = 0.005). Despite this increase, there was still a statistically significant difference between sham-operated and SCI + TPM groups (P < 0.001).

Histopathological and immunohistochemical changes
At 4 weeks post-injury, histopathological examination showed that necrosis, infiltration of inflammatory cells, demyelination, and vacuolation were observed in the SCI only group. These findings were less pronounced in the SCI + TPM group than in the SCI and SCI + vehicle groups. The ratio of the lesion area to the whole spinal cord section area was 36.2 ± 9.4%, 22.4 ± 8.4%, and 35.4 ± 8.1% in the SCI only, SCI + TPM, and SCI + vehicle groups, respectively. The lesion area was smaller in the SCI + TPM group than that in the SCI only and SCI + vehicle groups, although the difference did not reach statistical significance (P = 0.075 and P = 0.12, respectively; **Figure 3**). At 24 hours post-injury, iNOS, eNOS, and APAF-1 immunoreactivities were significantly different among the groups (P = 0.001). iNOS, eNOS, and APAF-1 immunoreactivities were significantly lower in the SCI + TPM group than in the SCI only and SCI + vehicle groups (all P = 0.001); however, iNOS, eNOS, and APAF-1 immunoreactivities in the SCI+ TPM group were significantly greater than those in the sham-operated group (all P = 0.001; **Figure 1E**).

Behavioral changes
The highest MFS and IPS values were detected in the sham-operated group (P = 0.01). MFS and IPS values in the SCI + TPM group were significantly higher than those in the SCI only and SCI+ vehicle groups (both P = 0.001); however, there was also a statistically significant difference between sham-operated and SCI + TPM groups (P = 0.001; **Figure 4**).

Discussion
SCI is a major public health problem resulting in motor, sensory, and autonomic dysfunction. The pathophysiology of SCI is complex and includes primary and secondary injury mechanisms. The severity of the primary injury varies with the amplitude and duration of the trauma energy and it is almost impossible to intervene with this process. Secondary injury is induced by primary mechanical insult and occurs because of a cascade of various pathophysiologic processes within days following the initial trauma (Dumont et al., 2001). These processes include inflammation, ischemia, edema, increased glutamate levels, free oxygen radicals and cell membrane damage, lipid peroxidation, vascular mediators and nitric oxide (NO) production, and activation of proapoptotic mediators (Azbill et al., 1997; Lu et al., 2000; Dumont et al., 2001; Park et al., 2004). Necrosis and apoptosis cause neuronal loss, destruction of the axon-myelin structure, and finally loss of function (Schwartz and Osborne, 1993).

We showed that TPM, an AMPA receptor inhibitor and widely used antiepileptic medication, provides effective biochemical, histological, and functional protection against SCI when delivered within 30 minutes after injury. To the best of our knowledge, this is the second study confirming the neuroprotective effects of TPM in SCI. In a previous report, in a model of cervical spinal cord contusion injury, Gensel et al. (2012) showed that TPM, if delivered 15 minutes after SCI, increases tissue sparing and preserves oligodendrocytes and neurons at 48 hours post-injury. They also showed that TPM was superior to NBQX, a well-documented neuroprotective AMPA receptor antagonist, in the protection of neurons and oligodendrocytes. The present study supports and adds new dimensions to this former study (Gensel et al., 2012). Initially, we showed that TPM also provides protection against oxidative damage after SCI as reflected by reduced lipid peroxidation and protein carbonylation and increased reduced GSH levels. Oxidative stress is a hallmark of secondary injury after spinal trauma (Azbill et al., 1997; Dumont et al., 2001; Vaziri et al., 2004; Jia et al., 2012). Thus, alleviating oxidative stress is considered as an effective therapeutic strategy for SCI. Two agents methylprednisolone and 21-aminosteroid tirilazad possess significant antioxidant activities and improve recovery of patients with SCI in clinical trials, whereas many others, including carotenoids and phenolic compounds, are also protective in experimental studies (Bilginer et al., 2009; Jia et al., 2012). Protection against oxidative damage by TPM, as shown in animal models of epilepsy, might be directly due to antioxidant properties and indirectly due to other mechanisms of action, taking into account the complex nature of pathophysiological processes involved in SCI (Cárdenas-Rodríguez et al., 2013; Naziroglu and Yürekli, 2013).

NO has a major role in secondary injury (Dawson et al., 1993; Estevez et al., 1998; Conti et al., 2007; Garry et al., 2015). Nitric oxide synthase (NOS) has three subtypes: neuronal NOS (nNOS), endothelial NOS (eNOS), and finally inducible NOS (iNOS) found in macrophages and glia. It has been demonstrated that NO can exert both protective and detrimental effects in SCI depending on several factors, such as level of produced NO, activity of different synthase isoforms, cellular source of production, and time of release (Satake et al., 2000; Dumont et al., 2001; Vaziri et al., 2004; Conti et al., 2007; Yang et al., 2007). High NO levels produced by upregulated nNOS and iNOS, several hours to days after the trauma, were found to be neurotoxic (Conti et al., 2007). Previous studies suggested that eNOS and iNOS have opposing roles in the pathophysiology of SCI. The expression of NOS subtypes increases during the initial days after spinal cord trauma (Vaziri et al., 2004; Conti et al., 2007; Yang et al., 2007). eNOS is known as a potent vasodilatator exhibiting neuroprotective effects after cerebral ischemia. Additionally, apoptosis is reduced by NO produced by eNOS (Estevez et al., 1998). Phosphorylation of nNOS might have an important role in regeneration because of increased blood flow at the lesion site (Osuka et al., 2007). Effects of iNOS on inflammation and apoptosis after SCI have been reported previously (Satake et al., 2000). Mediators, such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), secreted during SCI increase the expression of iNOS and result

in the upregulation of iNOS in macrophages and astrocytes (Dawson et al., 1993; Satake et al., 2000). High iNOS levels inhibit mitochondrial functions and stimulate apoptosis and excitotoxic neuronal death (Satake et al., 2000; Wu et al., 2007). In our study, we have found that following the trauma, eNOS and iNOS levels were increased at the lesion site, and they were diminished after administration of TPM. This finding shows that these two enzymes may together have roles in the pathophysiology of SCI. Further studies are needed to identify mechanisms by which TPM lowers NOS levels and whether this decrease indeed translates into a protective effect. We also found that the activity of APAF-1, an apoptotic marker (Shakeri et al., 2017), was lower in the SCI + TPM group than in SCI only and SCI + vehicle groups. This finding also suggested that TPM may directly or indirectly inhibit apoptosis.

For evaluation of the functional outcome after SCI, MFS and IPS were used as a measurement of neurological recovery. In our study, functional recovery was found in SCI rats following TPM treatment.

Being a broad spectrum anticonvulsant and having multi-mechanistic properties (Shank and Maryanoff, 2008), TPM might well be a promising therapy for treating SCI. In addition to antagonizing glutamate receptors, it enhances the effects of the inhibitory neurotransmitter (*i.e.*, GABA), reduces the activity of voltage-gated sodium and calcium channels, and blocks the influx of calcium into cells (Shank et al., 2000; Dinoff et al., 2003; Shank and Maryanoff, 2008).

Unlike other glutamate receptor antagonists that have failed clinical trials because of undesired side effects (Walters et al., 2005; Chen and Lipton, 2006), TPM is available clinically for migraine and epileptic seizure treatment and is well-tolerated by individuals with SCI (Shank et al., 2000; Dinoff et al., 2003; Shank and Maryanoff, 2008). Because it is clinically used to reduce pain related to SCI, systemic administration of TPM may prove effective for acute phase SCI treatment as well.

To conclude, in this study, biochemical, immunohistochemical, and neurobehavioral findings support the neuroprotective role of TPM in SCI. We found that TPM treatment diminished oxidative damage after SCI. Immunohistochemical parameters significantly subsided, except for eNOS levels, after TPM treatment in the acute phase of SCI. Additionally, TPM treatment also improved function after SCI. This study provides evidence for neuroprotective effects of TPM in SCI; however, further experimental and clinical studies are needed to explore its mechanism of action, evaluate its short- and long-term effects, establish an optimal dosing scheme, and assess its combination with other therapeutics.
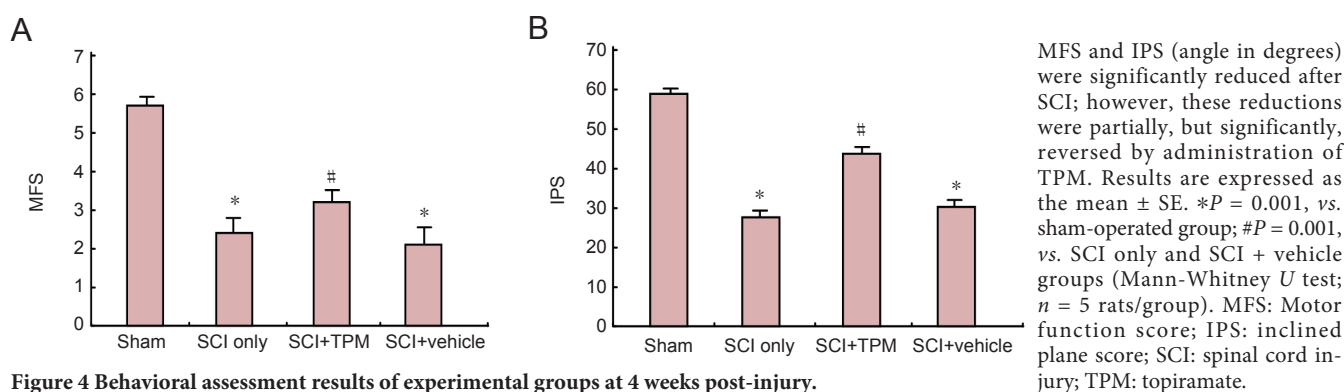
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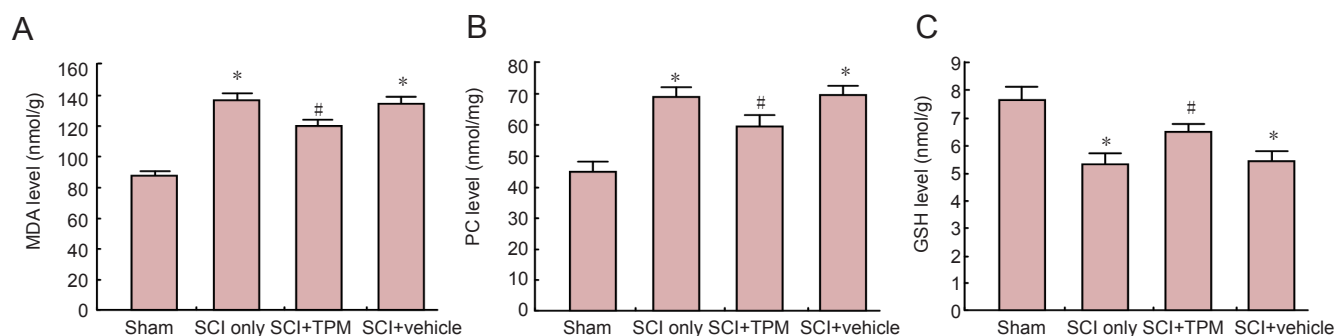
Figure 2 Biochemical assessment results of experimental groups at 24 hours after injury.
MDA (A), PC (B), and GSH (C) levels were determined after injury. Administration of TPM partially reversed these changes. Results were expressed as the mean ± SE. *$P < 0.001$, *vs.* sham(-operated) group; #$P < 0.05$, *vs.* SCI only and SCI + vehicle groups (Mann-Whitney U test; $n = 5$ rats/group). MDA: Malondialdehyde; PC: protein carbonyl; GSH: glutathione; TPM: topiramate; SCI: spinal cord injury.

MFS and IPS (angle in degrees) were significantly reduced after SCI; however, these reductions were partially, but significantly, reversed by administration of TPM. Results are expressed as the mean ± SE. *$P = 0.001$, *vs.* sham-operated group; #$P = 0.001$, *vs.* SCI only and SCI + vehicle groups (Mann-Whitney U test; $n = 5$ rats/group). MFS: Motor function score; IPS: inclined plane score; SCI: spinal cord injury; TPM: topiramate.

Figure 4 Behavioral assessment results of experimental groups at 4 weeks post-injury.
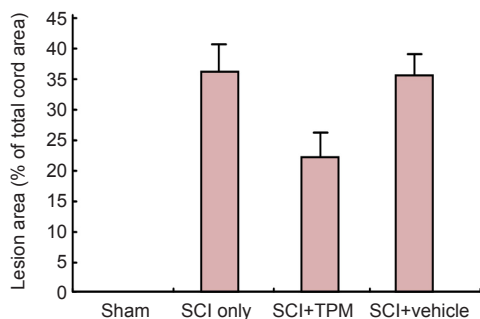
Figure 3 Spinal cord lesion area measurements of experimental groups at 4 weeks post-injury.
Lesion area was significantly less in the SCI + TPM group than in the SCI only ($P = 0.075$) and SCI+ vehicle ($P = 0.09$) groups ($n = 5$ animals/group). TPM: Topiramate; SCI: spinal cord injury.

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