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Epifriedelinol Ameliorates the Neuropathic Pain and Recovers the Function in Spinal Cord Injury by Downregulation of Neuronal Apoptosis and NMDA Receptor

Haiying Guan,[1] Ting Yan,[2] Dongyang Wu[3] and Alok Shiomurti Tripathi[4,5]

[1]Department of Spine Surgery, Xi'an Hospital of Traditional Chinese Medicine, Xi'an, China
[2]Department of Ultrasound, Xi'an Honghui Hospital, Xi'an, China
[3]Department of Neurosurgery, General Hospital of Northern Theater Command, Shenyang, China
[4]Department of Pharmacology, Amity Institute of Pharmacy, Amity University, Noida, Uttar Pradesh, India
[5]Kannur College of Pharmacy, Kannur, Kerala, India

Spinal cord injury (SCI) is commonly associated with neuropathic pain, which affects large population. Thus, the presented investigation evaluates the beneficial effect of epifriedelinol against SCI-associated neuropathic pain. SCI injury was induced in rats by clip-compression and rats were treated with epifriedelinol 100 and 200 mg/kg, i.p. for 21 days after the induction of SCI. The effect of epifriedelinol was assessed on neuropathic pain by mechanical allodynia and locomotor function. Level of inflammatory cytokines were assessed in the neuronal tissue using enzyme linked immunosorbent assay (ELISA) and expression of caspase-3 and Bcl2 protein were assessed by western blot assay. Data of investigation reveals that epifriedelinol reduces mechanical allodynia in SCI injured rats. Moreover, it also improves locomotor function in SCI injured rats. There was significant decrease in level of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α in the neuronal tissues of epifriedelinol-treated group than negative control group. Moreover, treatment with epifriedelinol ameliorates the altered expression of caspase 3, Bcl2 and GluN1 and level of glutamate in neuronal tissue of SCI-injured rats. In conclusion, data reveal that epifriedelinol treatment protects neuropathic pain associated with spinal cord injury by downregulating the N-methyl-D-aspartate (NMDA) receptor function.

Keywords: epifriedelinol; glutamate; NMDA; spinal cord injury
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Introduction

Spinal cord injury (SCI) is one of the common causes of disability among the Chinese population. SCI is associated with neuropathic pain with the loss of motor function (Shiao and Lee-Kubli 2018). Traffic accidents, fall from buildings and tall trees are the common causes of SCI reported clinically (Chen et al. 2016). SCI occurs in two different way; one is primary injury which occurs due to direct injury or trauma, and secondary injury occurs after the advancement of primary injury with complex pathogenesis such as ischemia, oxidative damage, degeneration of neurons by apoptosis/necrosis and excitotoxicity (Alizadeh et al. 2019). SCI leads to centrally acting neuropathic pain which required administration of central acting analgesic, and chronic administration of it has several limitations (Fornasari 2017). Therapeutic management of SCI needs better understanding of molecular pathway involved in it to develop novel therapy. Moreover, there is requirement of prevention of development of secondary injury. The Nmethyl-D-aspartic acid (NMDA) receptor contributes to the pain transmission as it sensitizes the central nervous system (Zhou et al. 2011). Moreover, NMDA receptor antagonist attenuates the pain transmission in experimental-induced SCI animal model and human (Miranda et al. 2014). However, the adverse effect of NMDA antagonist shows dysphoria and hallucination like severe side effect (Chen et al. 2009), and thus there is binding with specific subunit of NMDA receptor to provide minimum adverse events.

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Correspondence: Dongyang Wu, Department of Neurosurgery, General Hospital of Northern Theater Command, Shenyang 110015, China.
e-mail: dongyang3603@sina.com

Natural source molecule has shown potential for the management of disorders including SCI. Epifriedelinol is chemically a pentacyclic triterpene isolated from *Vitex peduncularis* Wall. ex Schauer belonging to Verbenaceae, and *Aster tataricus* belonging to Asteraceae (Ng et al. 2003). Epifriedelinol was reported to possess several pharmacological activities including anti-cancer, anti-inflammatory, anti-oxidant and anti-bacterial activities (Duke and Ayensu 1985; Kannathasan et al. 2019). Moreover, epifriedelinol protects neuronal injury by reducing oxidative stress and inflammatory cytokines in traumatic brain injured rats, as it reduces interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Li et al. 2018). Thus, the presented report evaluates the protective effect of epifriedelinol against neuroprotective effect against SCI rat model.

Materials and Methods

Animals

Sprague-Dawley rats (12-week-old male rats weighing 200-230 g) were housed under controlled conditions such as humidity (~60%), temperature (25 ± 2˚C) and 12 h light/dark cycle. All the experimental protocol were approved on July 15, 2018 from the institutional animal ethical committee of Kannur College of Pharmacy, India (2002/PO/Re/S/18/CPCSEA).

Establishment of spinal cord injury (SCI) rat model

SCI was induced in rats following the previously reported study (Kjell and Olson 2016). The rats were segregated randomly in four different groups: control group (without SCI), negative control group (with SCI), and two epifriedelinol-treated groups (treated with 100 or 200 mg/kg epifriedelinol, i.p. for 21 days after the induction of SCI). Briefly, pentobarbital at 35 mg/kg doses was intravenously injected to the rats for anesthesia. Laminectomy of the rats was performed for induction of SCI at T9-T10 level. The spinal cord in the region was exposed carefully to edema injury taking care that dura is not disrupted. The equal volumes of phosphate-buffered saline (PBS) were given to rats in the control and negative control groups. The rats were sacrificed on day 21st of surgery after anesthetization with pentobarbital for further experimental protocols (Fig. 1).

Assessments of behavior

Basso Beattie Bresnahan (BBB) scale was used to assess the locomotor function of SCI rats (Basso et al. 1995). Assessment of score was observed by two different researchers independently for each rat. Score 21 was marked for normal locomotion and score 0 recorded for paralysis rats.

Assessment of pain behavior

All the rats were assessed for mechanical allodynia by placing them on the plastic dome which covers metal mesh. Mechanical allodynia was observed before administration of drug and 15, 30, 60, 90, 120, 150, and 180 min after injection.

Von Frey filaments of 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 15.0, and 26.0 g were used to estimate the threshold for mechanical allodynia. Up-down method was used to stimulate the third metatarsal bone area of the left hindpaw using von Frey filaments. The minimum pressure required to activate a response was estimated to be the effect of epifriedelinol against mechanical allodynia in SCI injured rats.

Assessment of spinal cord edema

All the rats were sacrificed at the end of treatment protocol through decapitation method. Spinal cord from each rat were isolated from each rat and take the weight of wet spinal cord after washing it with PBS. Later dry weight of organ was recorded by drying the tissue at 72˚C for 48 h. The volume of spinal cord edema was calculated as follows: water content (%) = wet weight/dry weight × 100%.

Assessment of cytokine level

Spinal cord tissue isolated from each rat was homogenized under phosphate buffer, and levels of TNF-α, IL-1β and IL-6 were estimated in the tissue homogenate of SCI rat using ELISA method.

Western blot analysis

The spinal cord tissues harvested from rats were treated with RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) for preparation of lysate. Homogenization was performed for 30 min at 12,500 × g at 4˚C and protein content in supernatant was determined by bicinchoninic acid assay. The protein samples (30 μg) were loaded on 10% SDS-PAGE and subsequently transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were treated with 5% skimmed milk powder for 2 h to block the non-specific sites. Incubation with primary antibodies for probing of GluN1, caspase-3, Bcl-2 and β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA) were performed overnight at 4˚C. Membranes were washed, and subsequently incubated with horseradish per-

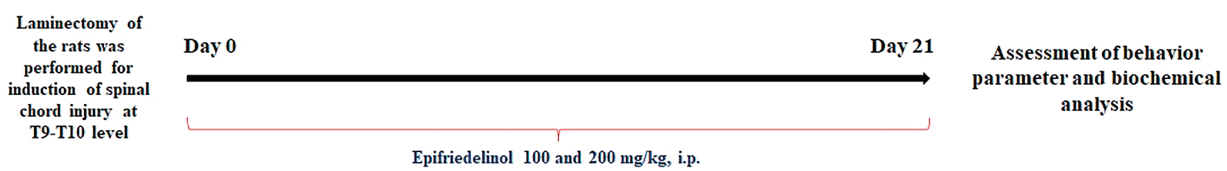

Fig. 1. Design of experimental protocol.

oxidase-conjugated secondary antibodies for 2 h at 37˚C. The immunopositive bands were detected using the BeyoECL Star and GeneTools software, version 4.1 (Synoptics, Ltd., Cambridge, UK) systems.

Statistical analysis

The data presented are expressed as mean ± standard error mean (SEM; n = 8). The data were analysed using SPSS, version 17.0 (SPSS, Inc., Chicago, IL, USA). The differences were determined statistically using one-way analysis of variance (ANOVA) and Tukey's test. At $p < 0.05$ differences were taken significant statistically.

Results

Epifriedelinol ameliorates the motor function

Motor function was estimated through BBB score to determine the effect of epifriedelinol in SCI injured rats on 0^{th} and 21^{st} day of protocol. BBB score was not altered in any of the group of animals on 0^{th} day of protocol. However, BBB score was reduced significantly ($p < 0.01$) in negative control group than control group of rats and treatment with epifriedelinol reverse the BBB score in SCI injured rats as shown in Fig. 2.

Epifriedelinol ameliorates pain behavior

Effect of epifriedelinol was estimated on the paw withdrawal threshold at different time interval such as pre, 15, 30, 60, 90, 120, 150 and 180 min after the administration of drugs in SCI injured rats. It was observed that paw withdrawal threshold enhances in epifriedelinol-treated group than negative control group. Moreover, epifriedelinol treatment improved the paw withdrawal threshold up to 21.33 g in SCI injured rats (Fig. 3).

Epifriedelinol ameliorates spinal cord edema

Effect of epifriedelinol was estimated on the spinal cord edema by determining the percentage of spinal cord contusion volume in SCI injured rats as shown in Fig. 4. Percentage of spinal cord contusion volume enhances significantly in negative control group than control group of rats. There was significant reduction in the percentage of spinal cord contusion volume in epifriedelinol-treated groups than negative control group, which means treatment with epifriedelinol reduces spinal cord oedema in SCI rats.

Epifriedelinol attenuates inflammatory cytokines

Inflammatory cytokines such as TNF-α, IL-1β and IL-6 were estimated in the spinal tissue of epifriedelinol-treated SCI rats as shown in Fig. 5. There were significant increases in cytokine levels in spinal tissue of negative control group than control group. Cytokine levels were reduced significantly ($p < 0.01$) in the spinal tissue of epifriedelinol-treated group than negative control group.

Epifriedelinol downregulates NMDA receptor

Effect of epifriedelinol was estimated on NMDA receptor by determining expression of GluN1 protein in the spinal tissue of SCI injured rats using western blot assay. There was significant increase in expression of GluN1 protein, which is subunit of NMDA receptor in spinal tissue of negative control group compared with control group of rats. Expression GluN1 protein was reduced significantly in the spinal tissue of epifriedelinol-treated group than negative control group (Fig. 6).

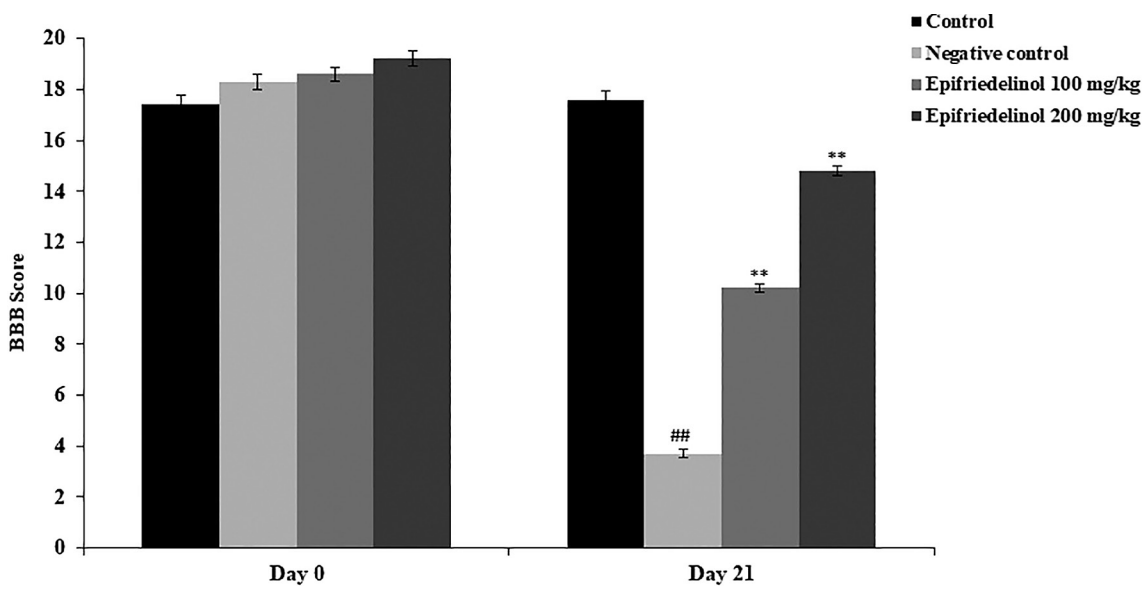

Fig. 2. Effect of epifriedelinol on the motor function by determining Basso Beattie Bresnahan (BBB) score in spinal cord injury (SCI)-injured rats.
Data are shown as Mean ± SEM (n = 8). $^{\#\#}p < 0.01$ control group vs. negative control group, $^{**}p < 0.01$ vs. negative control group.

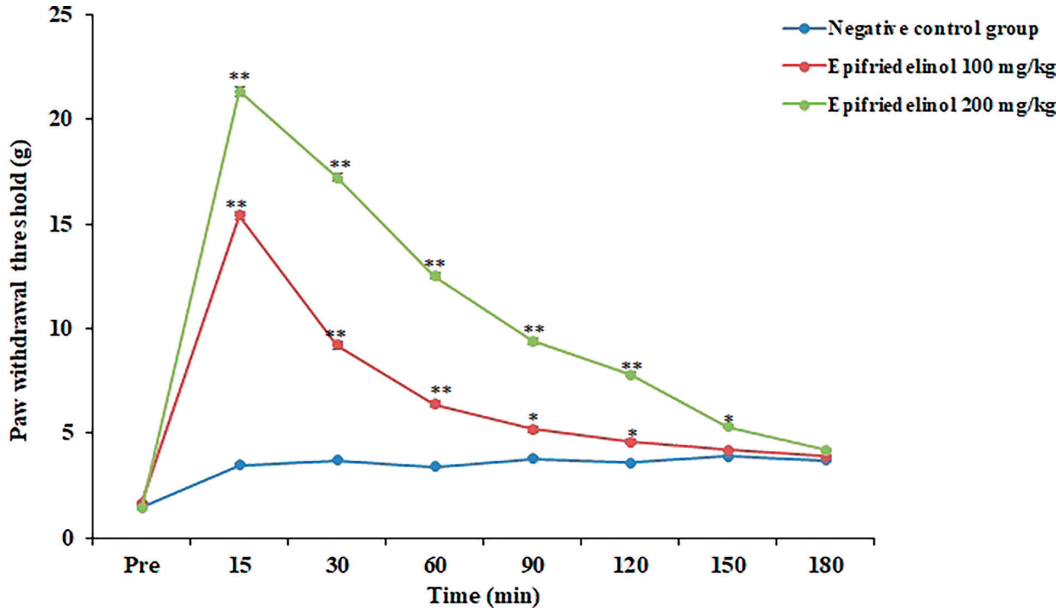

Fig. 3. Effect of epifriedelinol on the paw withdrawal threshold at different time interval in SCI-injured rats. Data are shown as Mean ± SEM (n = 8). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. negative control group.

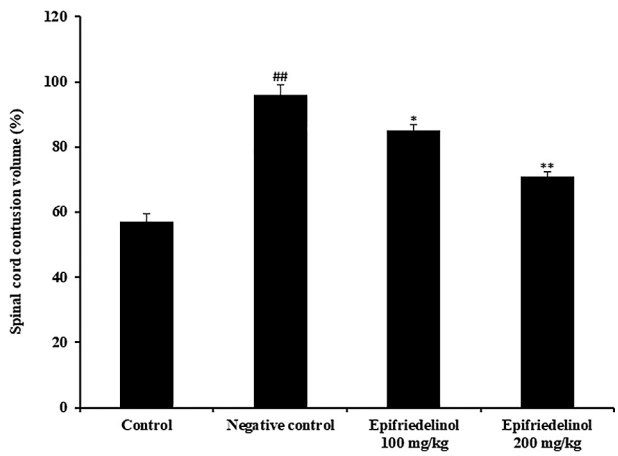

Fig. 4. Effect of epifriedelinol on the percentage of spinal cord contusion volume in SCI-injured rats. Data are shown as Mean ± SEM (n = 8). $^{\#\#}p < 0.01$ control group vs. negative control group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. negative control group.

Epifriedelinol protects neuronal apoptosis

Effect of epifriedelinol on the neuronal apoptosis was estimated by determining the expression of caspase-3 and Bcl-2 proteins in the spinal tissue of SCI injured rats using western blot assay. Enhanced expression of caspase 3 and reduced expression of Bcl-2 protein were observed in spinal tissue of negative control group than control group of rats. Treatment with epifriedelinol ameliorates the expression of caspase 3 and Bcl-2 proteins in the spinal tissue of SCI rats (Fig. 7).

Discussion

Spinal cord injury occurs due to traumatic injury to nerve ending of spinal cord, which alters the sensitivity of central nervous system, the distribution in pain sensitivity and the function, such as hyperalgesia and allodynia (Campbell and Meyer 2006). It is well documented that SCI enhances response towards the pain stimulus (Sawamoto et al. 2000). Moreover, secondary injury to the SCI leads to neuronal injury progression due to oxidative stress generation, ischemia, and excitotoxicity (Chen et al. 2016). Specifically, neuroprotective prevention of neuropathic pain is required for effective management of SCI. However, conventional drugs used clinically for the management of SCI have several serious limitations. Thus, there is a need to develop a novel therapeutic approach required for the management of SCI.

Literature reveals that laminectomy-induced SCI rat model resembles to clinical manifestation of SCI (Kjell and Olson 2016). Evidence report that SCI enhances odema and sensitivity (i.e., hyperalgesia) and allodynia like pain sensitivity (Jensen and Finnerup 2014). Data of the study reveal that the treatment with epifriedelinol reduces pain sensitivity and improves behavioral changes in SCI rats. Epifriedelinol possesses strong anti-inflammatory property and the present report also shows that edema to the spinal tissue reduces with its treatment in SCI rats. Inflammatory cytokines secretion associated with the cellular injury including with SCI (Zhang and An 2007). Traumatic SCI contributes to the increase in the level of cytokines, which enhances oxidative stress further leading to neuronal injury (Anjum et al 2020). Levels of IL-6, IL-1β and TNF-α enhance SCI, and contribute to further progression of neuronal injury through oxidative stress and increase in neuronal apoptosis (Wang et al 2015). Result of our report also supports that level of oxidative stress and cytokine (IL-6,

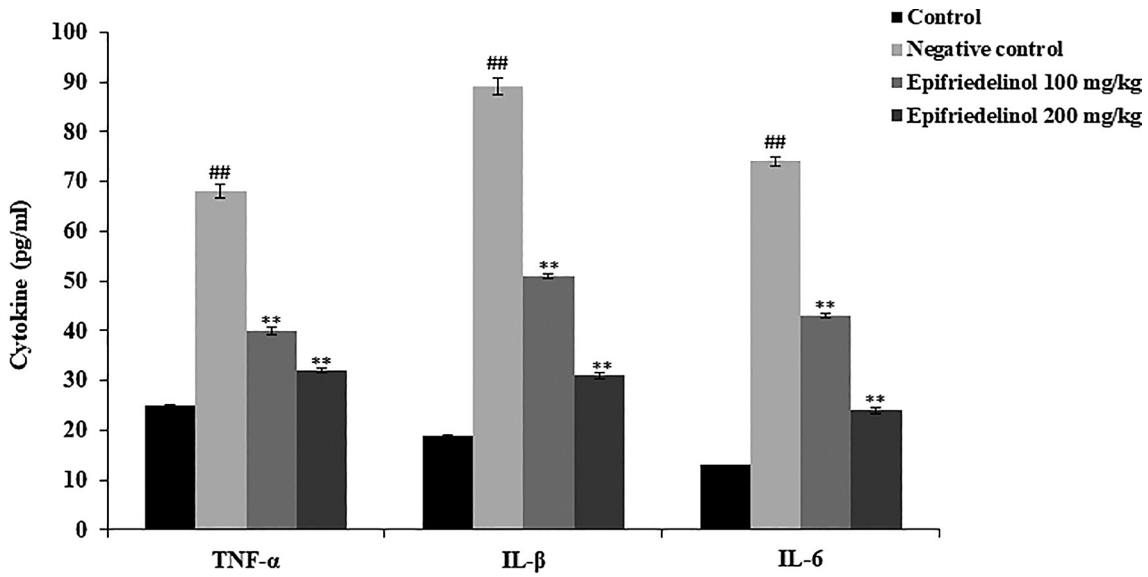

Fig. 5. Effect of epifriedelinol on the level of inflammatory cytokines in the spinal tissue of SCI-injured rats.
Data are shown as Mean ± SEM (n = 8). $^{\#\#}p < 0.01$ control group vs. negative control group, $^{**}p < 0.01$ vs. negative control group.

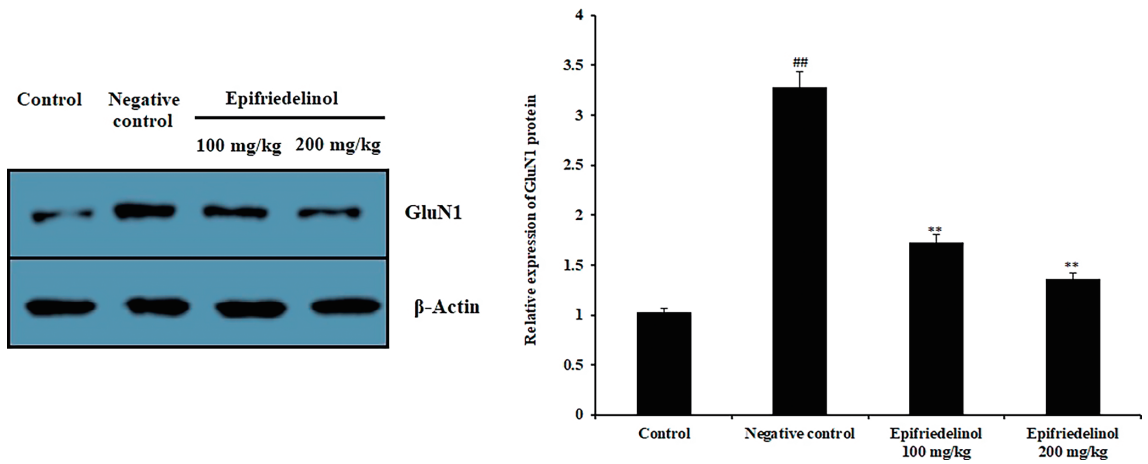

Fig. 6. Effect of epifriedelinol on the expression of GluN1 protein in the spinal tissue of SCI-injured rats.
Data are shown as Mean ± SEM (n = 8). $^{\#\#}p < 0.01$ control group vs. negative control group, $^{**}p < 0.01$ vs. control group.

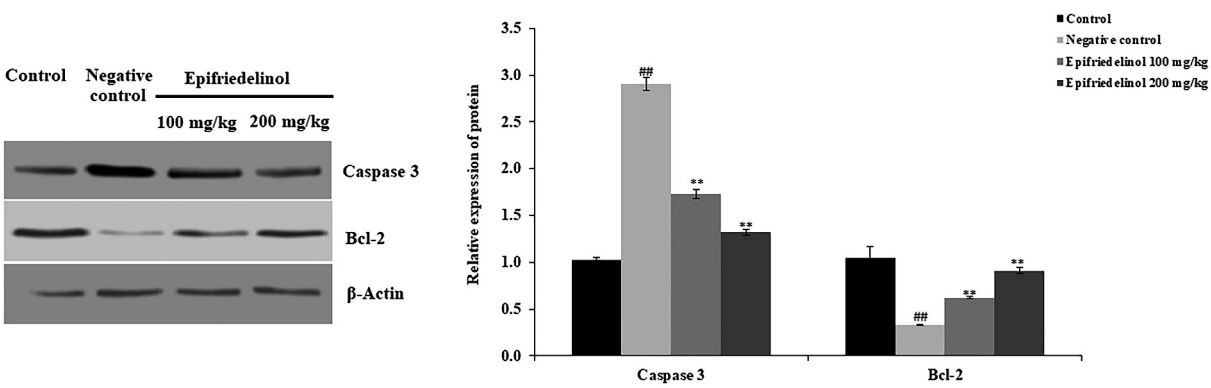

Fig. 7. Effect of epifriedelinol on the neuronal apoptosis in the spinal tissue of SCI-injured rats.
Data are shown as Mean ± SEM (n = 8). $^{\#\#}p < 0.01$ control group vs. negative control group, $^{**}p < 0.01$ vs. negative control group.

IL-1β and TNF-α) enhances in the spinal tissue of negative control group compared with control group of rats and the treatment with epifriedelinol reverses the altered level of oxidative stress and cytokines in SCI rats.

Neuronal excitability was reported to play neuropathic pain in SCI, due to NMDA receptor activity alteration (Inquimbert et al. 2018). It is well documented that transmission of nociceptive information in the spinal cord involves glutamate (Larsson 2009). There are several types of glutamate receptor which causes development of pathology of pain sensation. Cessation of activity of NMDA receptor or antagonizing the glutamate activity on NMDA receptor attenuates neuropathic pain against SCI (Kalia et al. 2008). NMDA receptor has subunit GluN1 and GluN2, and the reduction in expression of GluN1 reduces the activity of NMDA receptor (Shipton and Paulsen 2014). NMDA antagonist is suggested to reduce the GluN1, and data reveal that the treatment with epifriedelinol reduces expression of GluN1 protein. Moreover, excitotoxicity promotes neuronal injury by stimulating the activity of NMDA receptor, which was also reduced in epifriedelinol-treated groups.

In conclusion, the data of the present study reveal that the treatment with epifriedelinol reduces neuronal apoptosis and neuropathic pain in SCI rat model, as it regulates the expression of GluN1 by reducing inflammatory cytokines and oxidative stress. Results of the investigation suggest that epifriedelinol could be used clinically for the management of SCI.

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Author Contributions

Haiying Guan performed the experimental work and designed the rough draft of manuscript. Ting Yan contributed to experimental method and analysis of data. Dongyang Wu supervised and designed the experimental work. Alok Shiomurti Tripathi contributed to designing of manuscript and prepared the final draft of manuscript.

Conflict of Interest

The authors declare no conflict of interest.

References

Alizadeh, A., Dyck, S.M. & Karimi-Abdolrezaee, S. (2019) Traumatic spinal cord injury: an overview of pathophysiology, models and acute injury mechanisms. *Front. Neurol.*, **10**, 282.

Anjum, A., Yazid, M.D., Fauzi Daud, M., Idris, J., Ng, A.M.H., Selvi Naicker, A., Ismail, O.H.R., Athi Kumar, R.K. & Lokanathan, Y. (2020) Spinal cord injury: pathophysiology, multimolecular interactions, and underlying recovery mechanisms. *Int. J. Mol. Sci.*, **21**, 7533.

Basso, D.M., Beattie, M.S. & Bresnahan, J.C. (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. *J. Neurotrauma*, **12**, 1-21.

Campbell, J.N. & Meyer, R.A. (2006) Mechanisms of neuropathic pain. *Neuron*, **52**, 77-92.

Chen, S.R., Samoriski, G. & Pan, H.L. (2009) Antinociceptive effects of chronic administration of uncompetitive NMDA receptor antagonists in a rat model of diabetic neuropathic pain. *Neuropharmacology*, **57**, 121-126.

Chen, Y., Tang, Y., Allen, V. & DeVivo, M.J. (2016) Fall-induced spinal cord injury: external causes and implications for prevention. *J. Spinal Cord Med.*, **39**, 24-31.

Duke, J.A. & Ayensu, E.S. (1985) Medicinal Plants of China. Reference Publications, Inc., Algonac, MI.

Fornasari, D. (2017) Pharmacotherapy for neuropathic pain: a review. *Pain Ther.*, **6**, 25-33.

Inquimbert, P., Moll, M., Latremoliere, A., Tong, C.K., Whang, J., Sheehan, G.F., Smith, B.M., Korb, E., Athie, M.C.P., Babaniyi, O., Ghasemlou, N., Yanagawa, Y., Allis, C.D., Hof, P.R. & Scholz, J. (2018) NMDA receptor activation underlies the loss of spinal dorsal horn neurons and the transition to persistent pain after peripheral nerve injury. *Cell Rep.*, **23**, 2678-2689.

Jensen, T.S. & Finnerup, N.B. (2014) Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. *Lancet Neurol.*, **13**, 924-935.

Kalia, L.V., Kalia, S.K. & Salter, M.W. (2008) NMDA receptors in clinical neurology: excitatory times ahead. *Lancet Neurol.*, **7**, 742-755.

Kannathasan, K., Senthilkumar, A. & Venkatesalu, V. (2019) Crystal structure and antibacterial evaluation of epifriedelinol isolated from Vitex peduncularis Wall. ex Schauer. *Arab. J. Chem.*, **12**, 2289-2292.

Kjell, J. & Olson, L. (2016) Rat models of spinal cord injury: from pathology to potential therapies. *Dis. Model. Mech.*, **9**, 1125-1137.

Larsson, M. (2009) Ionotropic glutamate receptors in spinal nociceptive processing. *Mol. Neurobiol.*, **40**, 260-288.

Li, S., Zhang, Q. & Li, P. (2018) Protective effects of epifriedelinol in a rat model of traumatic brain injury assessed with histological and hematological markers. *Transl. Neurosci.*, **9**, 38-42.

Miranda, A., Mickle, A., Bruckert, M., Kannampalli, P., Banerjee, B. & Sengupta, J.N. (2014) NMDA receptor mediates chronic visceral pain induced by neonatal noxious somatic stimulation. *Eur. J. Pharmacol.*, **744**, 28-35.

Ng, T.B., Liu, F., Lu, Y., Cheng, C.H. & Wang, Z. (2003) Antioxidant activity of compounds from the medicinal herb Aster tataricus. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, **136**, 109-115.

Sawamoto, N., Honda, M., Okada, T., Hanakawa, T., Kanda, M., Fukuyama, H., Konishi, J. & Shibasaki, H. (2000) Expectation of pain enhances responses to nonpainful somatosensory stimulation in the anterior cingulate cortex and parietal operculum/posterior insula: an event-related functional magnetic resonance imaging study. *J. Neurosci.*, **20**, 7438-7445.

Shiao, R. & Lee-Kubli, C.A. (2018) Neuropathic pain after spinal cord injury: challenges and research perspectives. *Neurotherapeutics*, **15**, 635-653.

Shipton, O.A. & Paulsen, O. (2014) GluN2A and GluN2B subunit-containing NMDA receptors in hippocampal plasticity. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **369**, 20130163.

Wang, W.Y., Tan, M.S., Yu, J.T. & Tan, L. (2015) Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Ann. Transl. Med.*, **3**, 136.

Zhang, J.M. & An, J. (2007) Cytokines, inflammation, and pain. *Int. Anesthesiol. Clin.*, **45**, 27-37.

Zhou, H.Y., Chen, S.R. & Pan, H.L. (2011) Targeting N-methyl-D-aspartate receptors for treatment of neuropathic pain. *Expert Rev. Clin. Pharmacol.*, **4**, 379-388.