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Original Research Article

Tamoxifen inhibits astrocytic JAK2/STAT3 pathway in rats with traumatic spinal injury

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Abstract

Purpose: *To investigate the reducing effect of Morinda citrifolia capsules (MCC) on total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) in hypercholesterolemia patients.*
Methods: *This study was a randomized double-blind placebo-controlled clinical trial, with 60 subjects placed in two groups, viz, experimental (MCC group) and placebo (P group). The first group received two capsules of MCC (each capsule contains 500 mg extract) while the second group received two capsules of placebo (comprised of 500 mg fillers) 3 times daily, for 14 days for both groups. Overnight fasting cubiti venous blood (3 mL) was taken from each subject each time measurements were carried out. TC and LDL-C were measured by spectrophotometric assay using an automated analyzer.*
Results: *The results show that there was significant decrease in TC and LDL-C on day 14, compared to control (P group). Reduction in TC and LDL-C was 13.8 and 15.5 %, respectively. Decrease in TC and LDL-C levels was influenced by factors, such as age, BMI, exercise, diet, and smoking habits. In MCC group, the capsules significantly decreased TC levels ($p < 0.05$).*
Conclusion: *The results suggest that 1 g MCC, given orally thrice daily, significantly reduces TC and LDL-C levels in patients with hypercholesterolemia.*

Keywords: *Hypercholesterolemia, Cholesterol levels, Morinda citrifolia*

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INTRODUCTION

Spinal cord injury (SCI) affects several neuronal structures following mechanical injury [1]. Therefore, SCI has a complex pathophysiology. Because secondary injury after primary mechanical injury inhibits functional recovery in patients with SCI, research has been conducted for several years in an attempt to resolve this issue. Secondary injuries are triggered by inflammatory responses, which play a key role in the pathogenesis of SCI [2].

Any damage to the nervous system causes neuropathic pain (NP), which may compound other health problems, including diseases such as diabetes, surgical procedures, and traumatic injuries [3]. Studies have been conducted regarding NP treatment, which include the use of tricyclic antidepressants and calcium channel α_2-δ ligands; however, satisfactory results have not yet been achieved [3]. In recent years, it has been suggested that glial activation is sufficient for chronic pain sensitization [4], such that the spinal glia should be considered when treating NP. Glial activation results in an increase in neuromodulators, pro-inflammatory cytokines, and chemokines, along with growth factors that cause pain and hypersensitivity; neuronal excitability is also enhanced in the spinal dorsal horn (SDH) [5].

Various studies have shown that astrocyte activation depends on the phosphorylation state of Janus kinase (JAK) signal transducers and activators of the transcription 3 (STAT3) signaling pathway [6,7]. During activation of spinal astrocytes, inhibition of the JAK-STAT3 pathway can reduce the pain response, as well as peripheral nerve injury hyperexcitability in SDH neurons [8]. Inhibition of the cytokine signaling 3 (SOCS-3) protein results in feedback regulation such that the JAK-STAT3 pathway and NP are suppressed [8].

Tamoxifen has been shown to be a potent inhibitor of cell swelling in anion channels in experimental models of ischemic stroke [9, 10]. It has been reported that, along with the anion channels that are affected by the swelling of astrocytes, various pathways involved in central nervous system (CNS) damage are inhibited by tamoxifen [11]. Decreases in peroxynitrite levels in ischemia, along with removal of reactive oxygen species, have been attributed to tamoxifen [12]. It has been reported that anti-inflammatory responses in mouse and rat microglial cells are triggered by lipopolysaccharide-induced signaling cascades and activation of proteins [13].

In this study, we explored the inhibitory effects of intrathecal tamoxifen on neuroinflammation and the spinal astrocytic pathway following traumatic SCI in rats.

EXPERIMENTAL

Sprague-Dawley rats (220 – 250 g) were kept in a temperature-controlled environment (22 – 25 ºC) under a 12 h/12 h light/dark cycle, with free access to water and food. Every effort was made to minimize the suffering and pain of the animals; the experiments were performed according to the ethical guidelines of Changzhou Central Hospital. Written approval (no. CHC 130215) was obtained from the Ethical Committee of Changzhou Central Hospital for treatment of the experimental animals and the animal studies followed the guidelines of European Commission [14].

Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 2 % dimethyl sulfoxide (DMSO; 1 mg/mL) and administered intrathecally according to the guidelines established in previous reports [9,10]. The rats were divided into three groups: sham control (n = 21; only normal animals were included in this group); SCI (n = 21; only SCI was performed and normal saline was injected into the animals to make up for the shortfall in blood volume due to no drug being administered); and tamoxifen [n = 42; tamoxifen was administered at different concentrations to two subgroups (both n = 21; tamoxifen concentrations of 1 and 5 mg/kg/day) at different intervals].

It has been reported in previous studies that motor dysfunction has a pronounced effect on nociceptive behavioral test outcomes [15]. Therefore, rotarod testing was performed to evaluate the possible influence of intrathecal tamoxifen on motor function in the injured rats. For this purpose, rats were tested using an accelerating rotating rod (model 7650; Ugo Basile, Varese, Italy). Rats were placed on the rotarod after 1 min of training; the rod was linearly accelerated from 4 to 40 rpm over 5 min. The rats completed 3 runs at 10-min intervals, and the elapsed time before falling on each run was recorded for each rat individually at the outset, and then again for the different groups. After intrathecal administration of tamoxifen and saline at 30 min, the tests were performed daily for 7 d. The time taken by each rat to fall from the rotarod was noted and the final results were expressed as a percentage of each rat's baseline time.

The rats were anesthetized with pentobarbital (60 mg/kg) and transcardially perfused using 200 mL of 5 mM sodium phosphate buffered saline [PBS; 0.9 % (w/v); pH 7.3] and 500 mL 4 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The L5 spinal cord segments of the rats were harvested, immersed in 30 % sucrose overnight, and kept at 4 ºC. Transverse spinal sections were cut on a cryostat (Leica CM1800; Heidelberg, Germany) and rinsed three times in PBS at pH 7.4, with 10-min intervals between each rinsing. Then, sections were washed with 0.01 M PBS containing 10 % normal goat serum and 0.3 % Triton X-100 at 37 ºC for 1 h for blocking. After 2 h incubation at room temperature, further incubation at 4 ºC for 48 h was done with a mixture of anti-pSTAT rabbit immunoglobulin G (IgG) (1:1,000; Cell Signaling Technology, Danvers, MA, USA) in a mixture of PBS and 0.3% Triton X-100, 5 % (v/v) donkey serum (PBS-XCD), and 0.25 % λ-carrageenan. Then, washing was performed using 0.01 M PBS for 10 min, and sections were incubated at room temperature with a mixture of 10 µg/mL Alexa 488-conjugated donkey anti-rabbit IgG. All sections were fixed on glass slides after staining and covered using 50 % glycerol and 2.5 % triethylenediamine (as an anti-fading agent) in 0.05 M PBS. Sections were observed under a laser scanning microscope (FV 1000; Olympus, Tokyo, Japan) with appropriate settings for proper visualization and acquisition of clear digital images.

L5 spinal segments were dissected on ice and the left dorsal part of the spinal cord was homogenized. Segments were further split in a handheld pestle with sodium dodecyl sulfate (SDS) sample buffer (10 mL/mg/tissue) and a mixture of phosphatase and proteinase inhibitors (Sigma-Aldrich). The bicinchoninic acid (BCA) method was used for quantification of protein concentrations. Samples were boiled for 8 min in water and loaded onto an SDS polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) before being transferred a to polyvinylidene difluoride (PVDF) membrane (Immobilon™-P; Millipore, Billerica, MA, USA). A 5 % bovine serum albumin (BSA) solution was applied for 2 h to block membranes, and the following primary antibodies were applied at 4 ºC overnight: anti-pSTAT3 rabbit IgG (1 : 300; Cell Signaling Technology), rabbit anti-pJAK IgG (1 : 300; Cell Signaling Technology), and anti-β-actin mouse IgG (1 : 300; Sigma-Aldrich). Membranes were then incubated for 2 h with secondary antibodies, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1 : 5,000; Zhongshan, Beijing, China) and HRP-conjugated donkey anti-mouse IgG (1: 5,000; Zhongshan). The membranes were rinsed thrice (10 min per rinse) using Tris-buffered saline and Tween 20 (TBST). Protein blots were analyzed using Labwork software (Ultra-Violet Products, Cambridge, UK), and target protein levels were normalized against β-actin levels and compared among the experimental and control groups; intensity was quantified using densitometry.

An enzyme-linked immunosorbent assay (ELISA) was performed on all samples after isolating the left dorsal horns of the L5 spinal segments of all animals according to the method used for Western blotting. The amounts of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were measured using a Multi-Analyte ELISArray Kit (Mix-N-Match; SABiosciences, Frederick, MD, USA). The manufacturer's protocol was followed for all procedures.

Statistical analysis

The data are presented as mean ± standard deviation (SD). Analysis of the rotarod testing, Western blot and ELISA data were performed using SPSS software (ver. 16.0; SPSS Inc., Chicago, IL, USA) and by analysis of variance (ANOVA) with Bonferroni confidence interval adjustments. $P < 0.05$ was considered significant.

RESULTS

Nociceptive behavioral test performance can be impaired significantly by motor dysfunction in rats. However, in this study, intrathecal tamoxifen had no obvious effect on the motor function of rats after 30 min of administration relative to their own baselines (Figure 1).

An effect of intrathecal tamoxifen on the phosphorylation of STAT3 was observed; increased immunoreactivity was seen in rats with SCI administered tamoxifen compared with the sham controls, and comparatively weak immunoreactivity was observed in all subgroups when tamoxifen was administered after SCI (Figure 2).

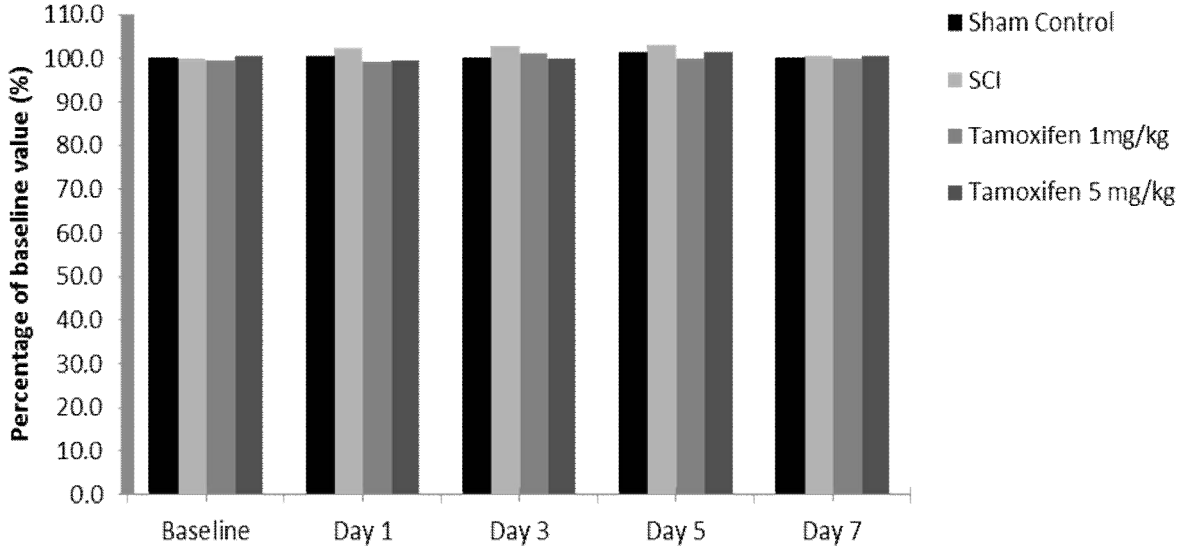

Figure 1: Effect of intrathecal tamoxifen on motor performance in the rotarod test Tamoxifen at 1 and 5 mg/kg did not have a pronounced effect on motor performance in rats with traumatic spinal cord injury (SCI) compared with the control group

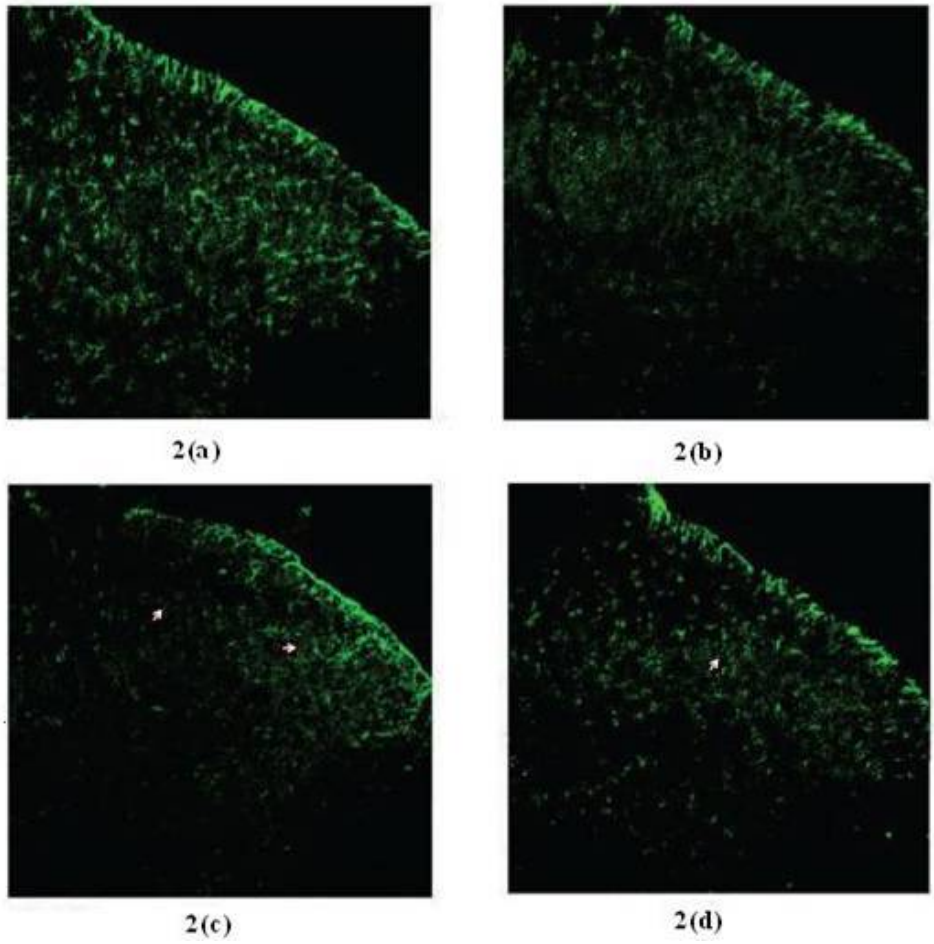

Figure 2: Effect of intrathecal tamoxifen on STAT3 phosphorylation in rats with SCI. (a) Increased pSTAT3 immunoreactivity was visible in rats with SCI; in these animals, there was an accumulation of pSTAT3 in spinal astrocytes. (b) Weak immunoreactivity of pSTAT3 was evident in normal controls. (c) Attenuation of pSTAT3 was evident in photomicrographs of rats treated with 5 mg/kg/day tamoxifen. (d) A comparatively less efficient pSTAT3 immunoreactivity result was evident in photomicrographs of rats treated with 1 mg/kg/day tamoxifen

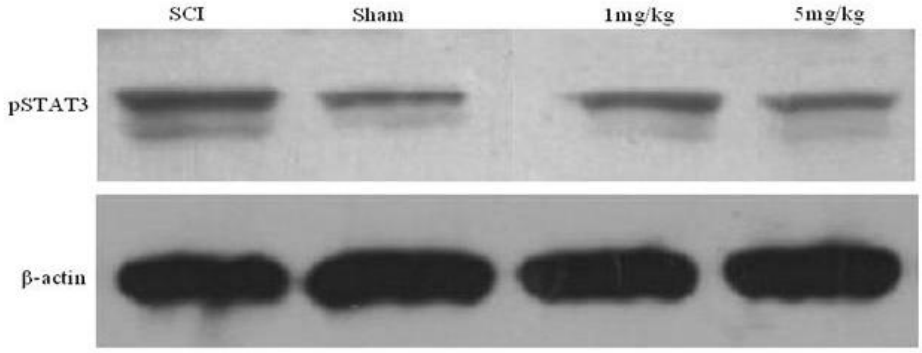

Figure 3: Western blot analysis showing the pSTAT3 levels of the different rat groups

To observe inhibition of the JAK2-STAT3 pathway after tamoxifen administration, JAK2 and STAT3 phosphorylation were examined using Western blot analysis. Significantly pJAK2 expression was significantly inhibited in tamoxifen-treated rats versus the sham control group ($p < 0.05$) (Figure 4).

elevated levels of pSTAT3 were seen in rats with SCI compared with rats that were treated with tamoxifen ($p < 0.05$) (Figure 3).

Elevated TNF-α, IL-1β, and IL-6 levels were present in rats with SCI than sham control group. However, treatment with tamoxifen significantly

($p < 0.05$) reduces the expression of proinflammatory cytokines than SCI group (Figure 5).

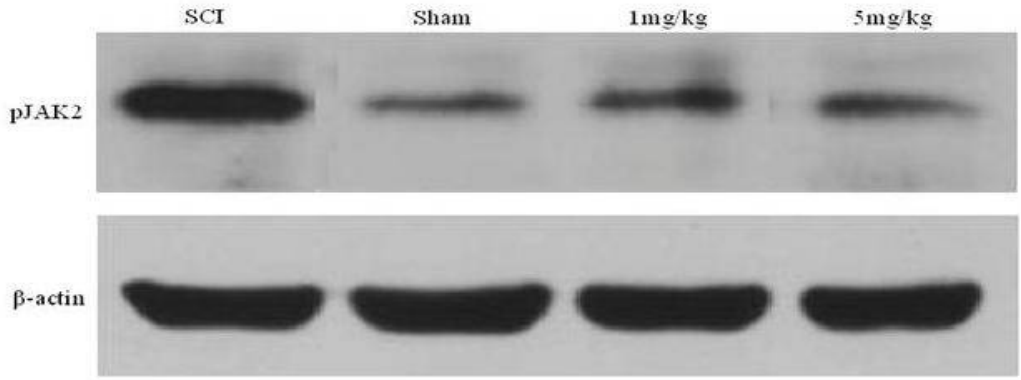

Figure 4: Representative Western blot analysis showing pJAK2 levels of the different rat groups.

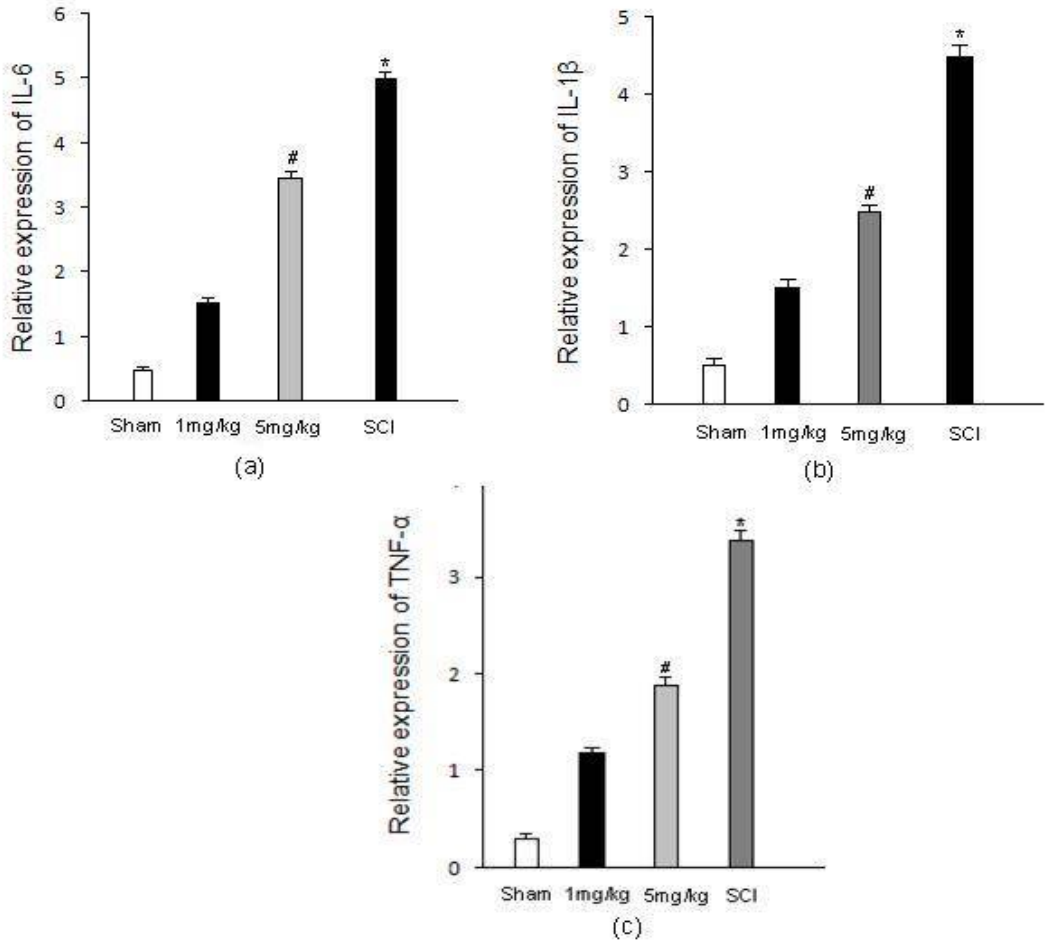

Figure 5: Effect of tamoxifen on pro-inflammatory cytokine levels. (a) Graphs showing decreased interleukin (IL)-6, (b) IL-1β, and (c) tumor necrosis factor (TNF)-α expression in tamoxifen-treated rats. $^{\#}P < 0.05$; $^{*}p < 0.05$

DISCUSSION

In this study, we observed that intrathecal administration of tamoxifen, at doses of 1 and 5 mg/kg/day, inhibits the proliferation of reactive astrocytes in the lumbar SDH; however, the 5 mg/kg/day dose was more effective. Astrocytic activation is regulated by activation of the JAK/STAT3 pathway; this activation was inhibited by administration of tamoxifen in injured rats, resulting in significantly reduced pJAK2 and pSTAT3 levels. A lower level of pro-inflammatory

cytokines was also observed, illustrating the anti-inflammatory effects of tamoxifen. Taken together, these results in rats with L4 and L5 injuries suggest that tamoxifen has good therapeutic potential.

Neuroprotective effects of tamoxifen have been reported in different studies and injury models. In an intracerebral hemorrhage model, a reduction in peri-hematomal brain edema was reported following use of tamoxifen [16], while reductions in pro-inflammatory cytokines and cell apoptosis in rats with SCI were reported by an earlier study [17]. In this study, we explored the possible role of tamoxifen in the JAK/STAT3 pathway in a spinal injury rat model.

Several studies have indicated that reactive astrocytes and microglia are important for maintaining NP [18]. Glial cells release different inflammatory stimulants, including neurotrophic factors, prostaglandins, and cytokines, which change the polarization of afferent neurons and result in the transmission of pain stimuli to the CNS [19]. Therefore, reducing pain and inflammation via a single agent has great therapeutic potential for treating spinal injuries in rats. We used 1 and 5 mg/kg/day tamoxifen doses based on traditional Chinese medicine regimens for treating different disorders.

It has been reported that the JAK-STAT pathway is an important signal transducer that responds to different external stimuli and transfers signals for cell migration, proliferation, and apoptosis from the cell surface to the cell nucleus [7]. Under conditions of astrogliosis, the JAK/STAT3 pathway plays a role as shown in models of NP, and brain ischemia [6,7].

The role of pro-inflammatory cytokines in spinal injuries has been well-established. It has been reported that proinflammatory cytokines IL-1β and TNF-α are important in initiating NP, which is an important and painful pathological condition in traumatic spinal cord injuries, while IL-6 is involved in maintaining NP [20,21].

CONCLUSION

The findings of this study show that proinflammatory cytokine levels are attenuated by tamoxifen at 5 mg/kg/day. This is consistent with inhibition of JAK/STAT3 pathway. Thus, tamoxifen at 5 mg/kg/day inhibits the JAK/STAT3 pathway, which in turn attenuates the expression of proinflammatory cytokines, eventually leading to anti-inflammatory effects as well as attenuation of NP in the rat model of SCI.

DECLARATIONS

Acknowledgement

Funding and research facilities were provided by the institution of the corresponding author.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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