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Ligustrazine attenuates neuropathic pain by inhibition of JAK/STAT3 pathway in a rat model of chronic constriction injury

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Aim: Neuropathic pain is a common clinical complication of nerve injury, and the effective treatment of neuropathic pain is still challenging. Ligustrazine is mainly used for the treatment of cardiovascular disease and its role in neuropathic pain is less investigated. The purpose of our study was to explore the effects of ligustrazine on neuropathic pain, as well as the underlying molecular mechanism. *Methods:* Neuropathic pain was induced by chronic constriction injury (CCI) of the right sciatic nerve in Sprague-Dawley (SD) rats. After CCI, rats received ligustrazine, IL-6, or both. Mechanical withdrawal threshold (MWT) and paw withdrawal thermal latency (PWTL) were assessed on days 1, 3, 7, and 14 after surgery. Expression levels of tumor necrosis factor (TNF)-α, interleukin (IL)-β, IL-2, and phosphorylation of Signal Transducer and Activator of Transcription (STAT) 3 were analyzed. *Results:* Our results showed that both MWT and PWTL were significantly decreased by CCI on days 1, 3, 7 and 14 compared to sham group, however, ligustrazine reversed this effects. Additionally, the elevated levels of TNF-α, IL-1β, and IL-2 in CCI spinal cord were inhibited by ligustrazine. Quantitative real-time (qRT-PCR) and Western blotting analysis showed that the test substance reduced the elevated expression of pSTAT3 in the spinal cord induced by CCI, and while IL-6 administration reversed the levels as well as the behavior responses. *Conclusion*: Our results suggest that ligustrazine could effectively attenuate neuropathic pain by inhibition of Janus Kinase (JAK)/STAT3 pathway in CCI rats.

1. Introduction

Neuropathic pain is caused by damage or dysfunction to the peripheral or central nervous system, which is characterized by a spontaneous hypersensitive pain response, continuous or paroxysmal (Butera 2007; Jensen et al. 2011). It has been estimated that approximately 6.9%-10% of the general population present neuropathic symptoms (van Hecke et al. 2014). Neuropathic pain not only significantly impairs quality of life, but also has a substantial economic impact on society (Doth et al. 2010; Gormsen et al. 2010; Smith and Torrance 2012; von Hehn et al. 2012). Despite some progress has been achieved in the past few years, an effective treatment is still unavailable due to the incomplete understanding of its pathogenesis. Therefore, the development of new, effective therapies for neuropathic pain is urgently required.

Recently, traditional Chinese medicine has received great attention to treat various diseases and is used extensively in research. Ligustrazine is an important active alkaloid extracted from *Ligusticum chuanxiong* Hort. Chuanxiong was first recorded in the Divine Husbandman's Classic of the Materia Medica (Shen Nong Ben Cao Jing), and has been reported to show numerous bioactivities, such as neuroprotection, anti-fibrosis, anti-nociception, antioxidant, vasorelaxation, anti-inflammation, anti-proliferation (Sun et al. 2008; Xiao et al. 2010; Ran et al. 2011; Kao et al. 2013). Besides, it displays some abilities of activating blood, promoting qi, expelling wind, and protecting against pain from the perspective of traditional Chinese medicine (Peng et al. 2009). Thus, it has been identified as an effective analgesic for injuries, including neuropathic pain. For example, Gao et al. (2008) reported that ligustrazine may suppress neuropathic pain by inhibiting the primary afferent transmission induced by $P2X_3$ receptor. Leng et al. (2012) suggested that ligustrazine could attenuate neuropathic pain-associated hyperalgesia and neuronal apoptosis in the spinal dorsal horn. However, whether ligustrazine effects are mediated by the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) signaling pathway is unclear.

In the present study, the effects of ligustrazine on neuropathic pain induced by sciatic nerve chronic constriction injury (CCI) were investigated, as well as the molecular mechanisms involved in JAK/STAT signaling pathway-induced neuropathic pain. Our study might provide new insights into new targets for treatment of neuropathic pain.

2. Investigations and results

2.1. Effects of ligustrazine on behavioral responses

Neuropathic pain was first induced by CCI. After CCI, rats were treated with ligustrazine (100 mg/kg, intraperitoneal, once daily) for 14 consecutive days. Mechanical withdrawal threshold (MWT) and paw withdrawal thermal latency (PWTL) were assessed on 0, 3, 7, and 14 days after surgery. As shown in Fig. 1 A and B, both MWT and PWTL were significantly decreased in the CCI group and CCI+ligustrazine group compared to the sham group 3 - 14 days after surgery ($P < 0.05$). There were no significant differences in both MWT and PWTL between the CCI group and the CCI+ligustrazine group on 0 and 3 days after operation; however, both MWT and PWTL in the CCI+ligustrazine group were significantly higher than the CCI group 3 - 14 days after surgery. The results suggested that administration of ligustrazine significantly improved behavioral responses related to neuropathic pain.

2.2. Effects of ligustrazine on expression of cytokines

It was well known that neuropathic pain is characterized by dysregulation of cytokines. Therefore, the effects of ligustrazine on expression of cytokines (TNF-α, IL-1β, and IL-2) were evaluated. The results showed that all the protein levels of TNF-α, IL-1β, and IL-2 were significantly increased in the spinal cord of CCI rats compared with the sham group ($P < 0.01$). However, ligustrazine reversed CCI-increased levels of TNF-α, IL-1β and IL-2 ($P < 0.05$), demonstrating

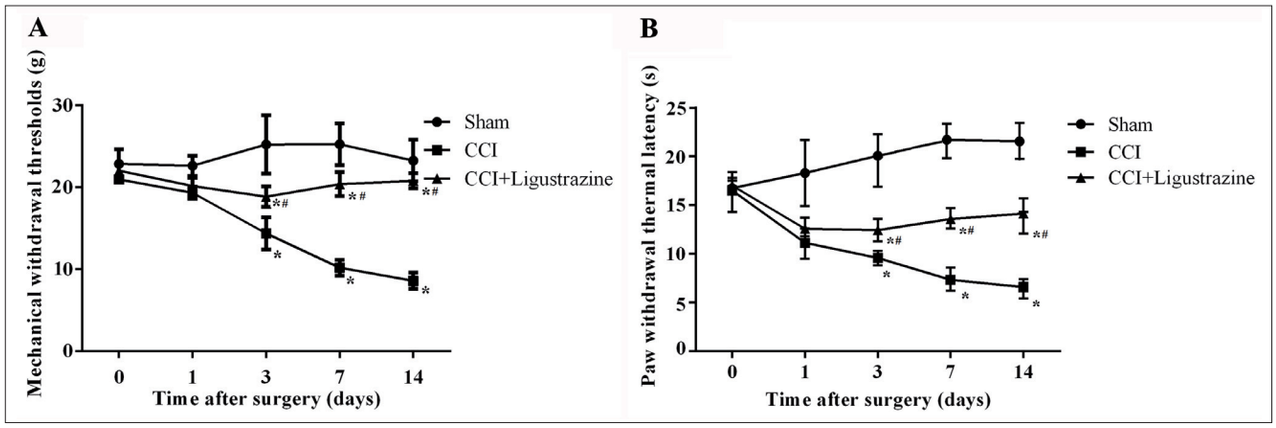

Fig. 1: Effects of ligustrazine on behavioral responses. A. Effects of ligustrazine on MWT; B. Effects of ligustrazine on PWTL. CCI, chronic systolic injury; MWT, mechanical withdrawal threshold; and PWTL, paw withdrawal thermal latency. $^{*}P < 0.05$ compared to the sham group; $^{\#}P < 0.05$ compared to the CCI group.

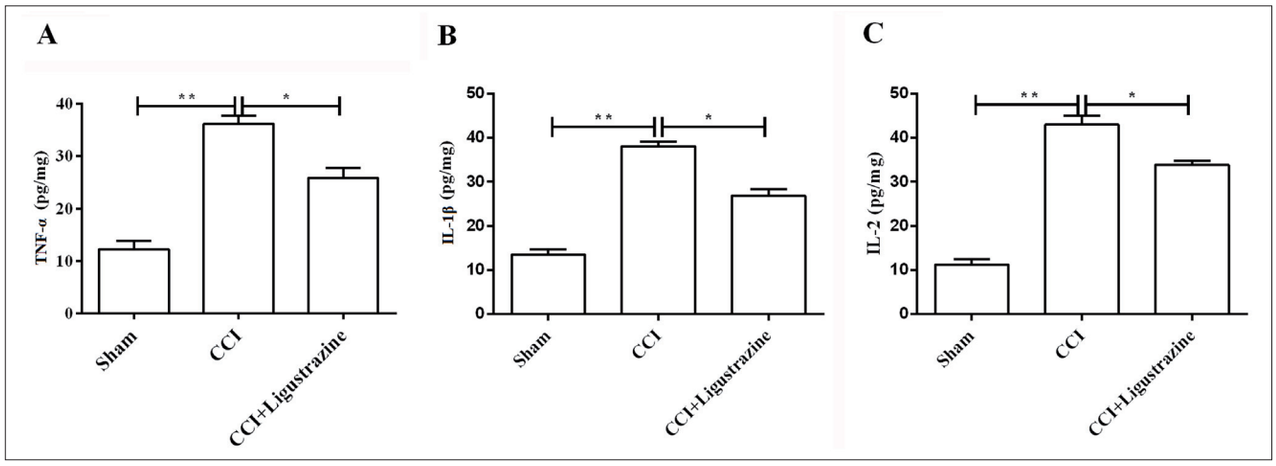

Fig. 2: Effects of ligustrazine on expression of cytokines. A. Effects of ligustrazine on expression of TNF-α; B. Effects of ligustrazine on expression of IL-1β; C. Effects of ligustrazine on expression of IL-2. CCI, chronic systolic injury; TNF, tumor necrosis factor; and IL, interleukin. $^{*}P < 0.05$ compared to the CCI group; $^{**}P < 0.01$ compared to the CCI group.

that ligustrazine reduced the expression of TNF-α, IL-1β, and IL-2 in spinal cord of CCI rats (Fig. 2 A-C).

2.3 Effects of ligustrazine on expression of p-STAT3

Previous studies have demonstrated that activation of the STAT3 pathway is responsible for the development of inflammation and neuropathic pain (Dominguez et al. 2008; Dominguez et al. 2010; Tsuda et al. 2011). Therefore, we hypothesized that ligustrazine might attenuate neuropathic pain by inhibition of the JAK/STAT3 pathway. As indicated in Fig. 3 A and B, both the relative mRNA and protein expression levels of p-STAT3 were significantly increased in the CCI group, but were markedly decreased by administration of ligustrazine ($P < 0.05$). Meanwhile, we applied IL-6, the major activator of JAK/STAT3, to the rats and investigated the effects. The results showed that treatment with IL-6 also markedly increased the levels of p-STAT3 compared to the CCI+ligustrazine group ($P < 0.05$). However, no significant differences were observed between the CCI group and the CCI+ligustrazine+IL-6 group. The results suggested that ligustrazine showed inhibitory effects on the JAK/STAT3 pathway. To confirm the results, we analyzed the behavior of rats. As indicated in Fig. 4 A and B, both MWT and PWTL were significantly decreased in the CCI group, but were markedly elevated by administration of ligustrazine. However, elevated MWT and PWTL after ligustrazine were significantly reduced by application of IL-6 (all $P < 0.05$). No significant differences in MWT and PWTL were found between the CCI group and the CCI+ligustrazine+IL-6 group, confirming that ligustrazine reduced neuropathic pain by inactivating the JAK/STAT3 pathway.

3. Discussion

In the present study, we investigated the effects of ligustrazine on neuropathic pain as well as the underlying mechanism. The main results of our study was that the CCI-decreased MWT and PWTL were reversed by ligustrazine. In addition, ligustrazine significantly inhibited the CCI-induced high levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-2). Furthermore, ligustrazine reduced the elevated levels of p-STAT3 in the spinal cord and abnormal behavioral responses induced by CCI. However, these effects were reversed by application of IL-6, a major activator of JAK/STAT3. Our results confirm that ligustrazine could effectively attenuate neuropathic pain by inhibition of the JAK/STAT3 pathway.

Neuropathic pain refers to the damage or disease involving the central or peripheral nervous system. It is often refractory to existing therapies. Currently, the main clinical medications for neuropathic pain include topical medications, antiepileptic medications, antidepressant medications, and opioids (Colombo et al. 2006). Although these drugs have been shown to be effective in relieving some symptoms of neuropathic pain, side effects are very common. This study was mainly done to explore the effects of

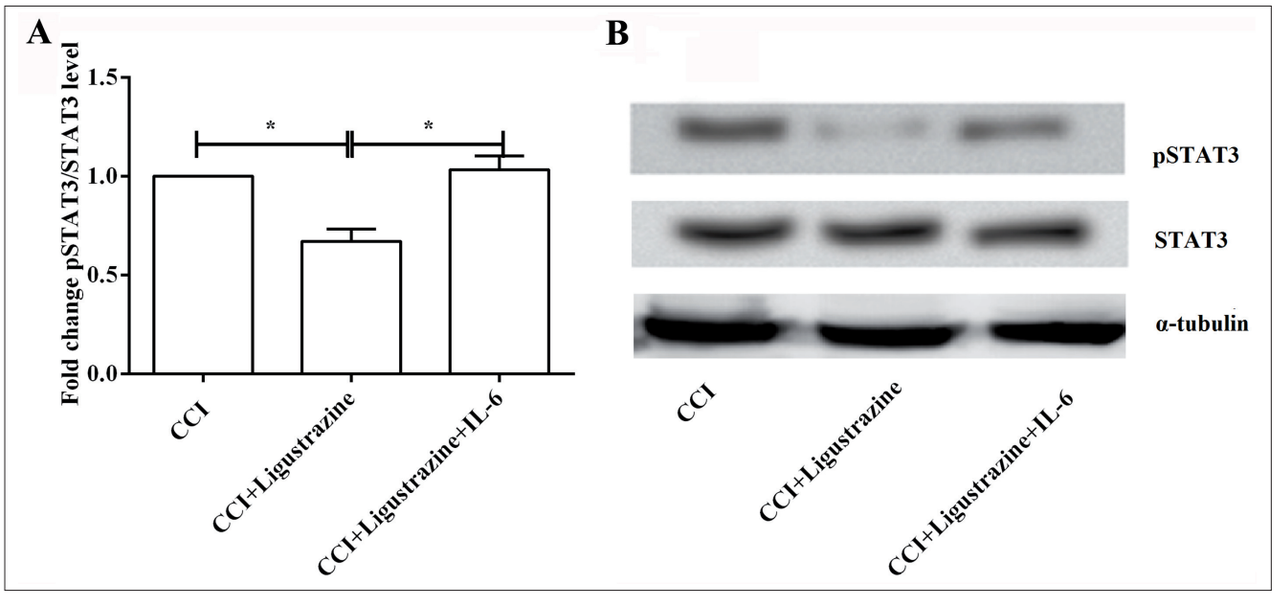

Fig. 3: Effects of ligustrazine on expression of p-STAT3. A, Fold change pSTAT3/STAT3; B, representative pictures of Western blotting. $^{*}P < 0.05$ compared to the CCI+ligustrazine group. CCI, chronic systolic injury; and STAT, Signal Transducer and Activator of Transcription.

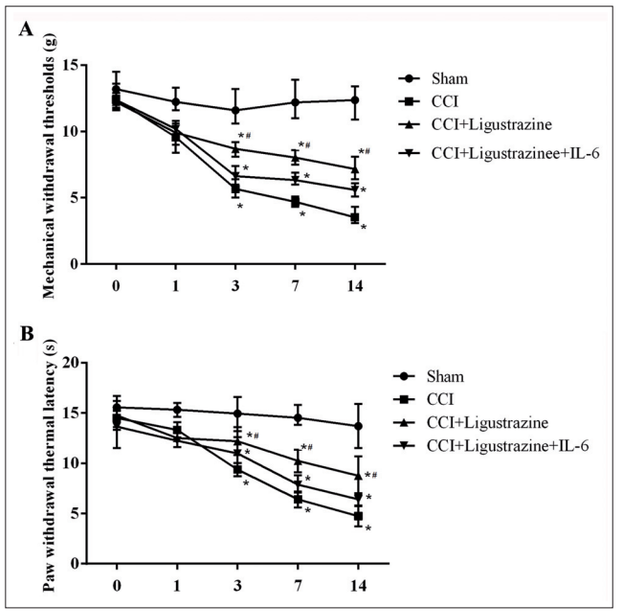

Fig. 4: Effects of ligustrazine combined with IL-6 on behavioral responses. A. Effects of ligustrazine combined with IL-6 on MWT; B. Effects of ligustrazine combined with IL-6 on PWTL. CCI, chronic systolic injury; MWT, mechanical withdrawal threshold; PWTL, paw withdrawal thermal latency; and IL, interleukin. *P < 0.05 compared to the sham group; #P < 0.05 compared to the CCI group.

ligustrazine, a traditional Chinese medicine, on neuropathic pain. Neuropathic pain was induced by CCI which is the most commonly used model because of its similarities with human behavioral responses (Wang and Wang 2003). We found that the mechanical allodynia and thermal hyperalgesia were significantly produced by CCI, which demonstrated that the model was successfully established. Additionally, we observed that ligustrazine markedly attenuated mechanical allodynia and thermal hyperalgesia induced by CCI, indicating the possible treatment effect of ligustrazine.

It has been well demonstrated that ligustrazine exerts an anti-inflammatory activity during the early and late stages of inflammation (Ozaki 1992). Ligustrazine has been shown to suppress the overexpression of pro-inflammatory cytokines TNF-α (Feng et al. 2011; Kao et al. 2013), IL-1β (Dang et al. 2007; Kao et al. 2013), and IL-2 (Cai et al. 2012). In addition, pro-inflammatory cytokines have been reported to be involved in neuropathic pain by demyelination, degeneration of peripheral nerves, and increase of sensory afferent excitability (Moalem and Tracey 2006). For example, pro-inflammatory cytokines TNF-α, IL-1β, and IL-2 have been suggested to play a critical role in the development and maintenance of neuropathic pain by directly and indirectly activating neurons (Moalem et al. 2004; Nadeau et al. 2011). Therefore, reduction of cytokines is considered as a potential target in the management of neuropathic pain after injury (Nadeau et al. 2011). In our study, the levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-2 were also determined. As indicated in our results, we found that all the levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-2 were significantly elevated in CCI rats, which was in line with previous studies. But these effects were reversed after application of ligustrazine to the CCI rats, confirming the anti-inflammatory effect of ligustrazine.

Besides, injury-induced neuropathic pain alters the signal transmission of pain involving a variety of intracellular signal transduction pathways. Among the intracellular signaling pathways, JAK/STAT3 has been suggested to be associated with neuropathic pain (Tsuda et al. 2011). Blockade of JAK/STAT3 signaling pathways by intrathecal administration of a JAK2 inhibitor or a STAT3 inhibitor reduces neuropathology, the upregulation of pro-inflammatory cytokines, and pain-related behavior (Tang et al. 2012; Wang et al. 2014; Xue et al. 2014). Therefore, we hypothesized that ligustrazine prevents neuropathic pain by involvement in the JAK/STAT3 signaling pathways. The levels of STAT3 and pSTAT3 were evaluated by qRT-PCR and Western blotting. Our results confirmed the elevated levels of pSTAT3 in CCI rats. The higher levels pSTAT3 were significantly decreased by administration of ligustrazine, suggesting that reduction of neuropathic pain by the compound might be due to an inhibition of the JAK/STAT3 signaling pathways. To verify this hypothesis, we gave IL-6, a major activator of JAK/STAT3 (Levy and Darnell 2002; Schindler et al. 2007), and then determined the levels of pSTAT3 as well as the behavior responses. The results showed that the levels of pSTAT3 were markedly elevated by a combination of ligustrazine and IL-6. Moreover, combination of ligustrazine and IL-6 showed no effect on alleviating pain-related behaviors. These results indicated that ligustrazine abolishes the activation of JAK/STAT3 induced by IL-6.

In conclusion, the present study suggests that ligustrazine could be a new agent in the clinical treatment of neuropathic pain. The protective role of ligustrazine in neuropathic pain might be associated with inactivation of JAK/STAT3.

4. Experimental

4.1. Animals

Adult male Sprague-Dawley (SD) rats, weighing 200-220 g, were obtained from experimental animal breeding center of our institute at 6-7 weeks of age. The animals were housed in separate group cages in a temperature (22±2 °C) and humidity (55±5 %) controlled room under a 12-h light-dark cycle. All the animals were provided food and water *ad libitum* and were acclimatized for one week before the experiment. The present study was approved by the Animal Care and Use Committee of Linyi People's Hospital.

4.2. Experimental design

All the animals were first randomly divided into 3 groups (n = 10 in each group): sham group, CCI group, and CCI+ligustrazine (100 mg/kg) (Sigma, St. Louis, MO, USA) group. Rats in the CCI+ligustrazine group were subjected to CCI and then received daily intraperitoneal injection of 100 mg/kg ligustrazine for two consecutive weeks after surgery, and while rats in the sham group and CCI group received equal volumes of dimethyl sulfoxide (DMSO). To further confirm the results, another group was submitted to the same schedule as those in the CCI+ligustrazine group, but received intrathecal IL-6 administration (1 μg).

4.3. Induction of neuropathic pain

Neuropathic pain was induced by CCI of right sciatic nerve (Bennett and Xie 1988). Briefly, the rats were anesthetized with 40 mg/kg sodium pentobarbital by intraperitoneal injection. The right sciatic nerve was exposed at the midthigh level. Four ligatures of 4.0 chromic catguts were tied loosely at 1.0 mm intervals. Then the incision was closed layer by layer. Meanwhile, a sham surgical procedure was carried out but without ligatures. After operation, all the rats were returned to their cages and kept under standard conditions.

4.4. Assessment of mechanical withdrawal threshold (MWT)

Mechanical hyperalgesia was determined by measuring the withdrawal thresholds to mechanical stimuli at 0, 3, 7, and 14 days after surgery using von Frey filaments (Stoelting Company, USA) as described previously (Chaplan et al. 1994). Briefly, the animals were placed in an individual Plexiglas chamber (23×18×13 cm) with a wire mesh floor and allowed to acclimatize for 30 min prior to this testing. The right hind paw of each rat was stimulated with ascending bending force) and held for < 6 s. Quick withdraw reflex of the right hind paw or removal of the paw was regarded as a positive response. Stimulation was started at 2.0 g. If there was no response, a filament with the next higher force was applied, but if there was a positive response, a filament with the next lower force was performed. The bending force that triggered three hind paw withdrawals was recorded, and the average value was calculated as MWT.

4.5. Assessment of paw withdrawal thermal latency (PWTL)

Thermal hyperalgesia was tested at 0, 3, 7, and 14 days after surgery using a Hargreaves apparatus (IITC, Woodland Hills, CA) according to the method previously described (Hargreaves et al. 1988). The animals were placed individually in a Plexiglas chamber and allowed to acclimate for 0.5 h before observations began. After acclimation, a radiant heat source was positioned underneath the platform directly beneath the right hind paw of each rat. The time between the application of the light beam and the rat took to lift its paw was recorded and considered as PWTL. A 25 s cutoff limit was imposed to prevent tissue damage.

4.6. Tissue preparation

Two weeks after CCI surgery, rats were killed after the last administration of drugs deeply anesthetized with 10% chloral hydrate (400 mg/kg intraperitoneal). The L4 - L6 sections of the spinal cord were removed immediately, washed with phosphate buffered solution (PBS), and then stored at -80 °C for further analysis.

4.7 Quantitative real-time (qRT-PCR)

Total RNA was extracted from spinal cord tissues using the RNAiso plus kit (TaKaRa, Shiga, Japan) according to the manufacturer's directions. The isolated RNA was then reverse-transcribed to produce the first strand complementary DNA (cDNA) with the cDNA synthesis kit (GIBCO/BRL; Life Technologies, Grand Island, NY) by using the oligo (dT) 18 primer. PCR amplification was carried out with an ABI 7900HT fast real-time PCR system using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA). All values were normalized to GAPDH mRNA with the comparative $2^{-\Delta\Delta CT}$ methods. All measurements were performed in triplicate.

4.8. Western blotting

Western blotting was performed to quantify the expression of phosphorylation of STAT3 and STAT3. Proteins were extracted from frozen spinal cord tissue pieces using RIPA lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) according to the manufacturer's instructions. The protein concentration was assessed by using a BCA protein assay kit (Pierce ThermoFisher, Rockford, IL, USA). The proteins were resolved on sodium dodecyl sulfonate (SDS) - polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane (Bio-Rad), blocked with 5% nonfat milk in Tris-Buffered Saline (TBS) containing 0.1 % Tween 20, and then incubated with anti-pSTAT3 antibody (1:1,000; Cell Signaling Technology) or anti-STAT3 antibody (1:1,000; Cell Signaling Technology) at 4 °C overnight. Thereafter, the blots were washed three times with PBS and incubated with horseradish peroxidase-conjugated secondary antibody. Membranes were then visualized with enhanced chemiluminescence (ECL) Plus kit (GE Healthcare, Piscataway, NJ, USA). Relative expression of the pSTAT3 and STAT3 was compared with the α-tubulin.

4.9. Enzyme-linked immunosorbent assay (ELISA)

Frozen spinal cords were homogenized in 10 mM Tris pH 7.4 (200 μL, 50 mM NaCl, 2.5 mM $MgCl_2$) supplemented with protease inhibitor cocktail. Then the samples were centrifuged at 4000 rpm for 15 min at 4 °C. The supernatant was stored at 80 °C for further protein quantification. The contents of tumor necrosis factor (TNF)-α, interleukin (IL-1) β, and IL-2 were analyzed by ELISA kits (Milipore Corporation, Billerica, MA, USA) according to the manufacturer's protocols. All samples were done in triplicate.

4.10. Statistical analysis

All collected data were shown as the mean±standard error of the mean (SEM). Statistical analysis was performed using Statistic Package for Social Science (SPSS) 17.0 software (SPSS, Chicago, IL). The statistical significance of differences in the behavioral data were analyzed by two-way analysis of variance (ANOVA) followed by Turkey's test, and while the differences in the biochemical tests were analyzed with one-way ANOVA followed by LSD *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

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