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Aloperine attenuated neuropathic pain induced by chronic constriction injury via anti-oxidation activity and suppression of the nuclear factor kappa B pathway

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ABSTRACT

Objective: To investigate whether aloperine (ALO) has antinociceptive effects on neuropathic pain induced by chronic constriction injury, whether ALO reduces ROS against neuropathic pain, and what are the mechanisms involved in ALO attenuated neuropathic pain.
Methods: Mechanical and cold allodynia, thermal and mechanical hyperalgesia and spinal thermal hyperalgesia were estimated by behavior methods such as Von Frey filaments, cold-plate, radiant heat, paw pressure and tail immersion on one day before surgery and days 7, 8, 10, 12 and 14 after surgery, respectively. In addition, T-AOC, GSH-PX, T-AOC and MDA in the spinal cord (L4/5) were measured to evaluate anti-oxidation activity of ALO on neuropathic pain. Expressions of NF-κB and pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) in the spinal cord (L4/5) were analyzed by using Western blot.
Results: Administration of ALO (80 mg/kg and 40 mg/kg, i.p.) significantly increased paw withdrawal threshold, paw pressure, paw withdrawal latencies, tail-curling latencies, T-AOC, GSH-PX and T-SOD concentration, reduced the numbers of paw lifts and MDA concentration compared to CCI group. ALO attenuated CCI induced up-regulation of expressions of NF-κB, TNF-α, IL-6, IL-1β at the dose of 80 mg/kg (i.p.). Pregabalin produced similar effects serving as positive control at the dose of 10 mg/kg (i.p.).
Conclusion: ALO has antinociceptive effects on neuropathic pain induced by CCI. The antinociceptive effects of ALO against neuropathic pain is related to reduction of ROS, via suppression of NF-κB pathway.

1. Introduction

Neuropathic pain (NP) is defined by the International Association for the Study of Pain (IASP) as "Pain caused by a lesion or disease of the somatosensory nervous system" [1]. Neuropathic pain is a major chronic pain condition that remains difficult to treat and a common condition with an overall prevalence between 0.9% and 8.0% [2–4]. Also, it can be categorized as central and peripheral neuropathic pain. Neuropathic pain associated with peripheral nerve injury is clinically well characterized by various sensory abnormalities such as spontaneous pain, hyperalgesia (an increased response to painful stimuli) and allodynia (pain in response to a stimulus that does not normally provoke pain) [5]. Previous studies suggested that individuals with NP were known to experience more severe pain compared to non-NP sufferers [3]. Neuropathic pain which afflicted people worldwide severely affected the quality of life, reduced individual productivity and increased patient and healthcare resource expenditure [6,7]. This serious phenomenon indicates that innovative treatment strategies are needed to control this disease.

Sophora alopecuroides L. (Leguminosae) is a commonly used traditional Chinese herbal, which widely distributed in the northwestern region of china and commonly used as antipyretic, antipyretic, anti-inflammatory and analgesic [8]. Aloperine (ALO), one of alkaloids isolated from *S. alopecuroides* L, possess a variety of pharmacological activities. Recently, studies have found that aloperine

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possessed anti-inflammatory, anticancer, anti-microbial, antiviral and anti-allergic effects [9–11]. However, its analgesic potential on neuropathic pain has been no reported. Therefore, the current studies were undertaken to explore the antinociceptive effects of ALO on neuropathic pain.

To study the mechanisms of neuropathic pain, a large of animal nerve injury models have been developed [12–17]. But chronic constriction injury (CCI) model was a widely employed for induction of neuropathic pain in experimental animals [12]. Extensive studies have demonstrated that ROS played an important role in neuropathic pain [18,19]. At the same time, NF-κB and its downstream pro-inflammatory cytokines which included TNF-α, IL-6, IL-1β also played a vital role in neuropathic pain [20]. Along this line, we speculated that anti-oxidation activity and NF-κB pathway may be involved in ALO attenuated neuropathic pain. In addition, the novel compounds pregabalin (Lyrica) which is a selective Cav 2.2 (a2-δsubunit) channel antagonist have been proven clinical efficacy in neuropathic pain [21,22] and served as positive control in this study. Therefore, the present studies were undertaken to determine the above speculations.

2. Materials and methods

2.1. Experiment animals

Male ICR mice weighing 18–22 g were obtained from the Experimental Animal Center of Ningxia Medical University (Certificate number was SYXK Ningxia 20050001). The animal house temperature was controlled at 22–24 °C and the relatively humidity of the house was kept at 45–65% under a 12 h light and dark cycles. The experimental protocol was duly approved by the institutional animal ethics committee of Ningxia Medical University, Yinchuan city, Ningxia. This study complied with the internationally accredited guidelines and ethical regulations on animal research.

2.2. Compounds

Aloperine (purity ⩾ 98.0%), Sodium pentobarbital and pregabalin were purchased from Ningxia Zi Jing Hua Pharmacy, Yinchuan Ningxia, Sigma-Aldrich, Steinheim, Germany and Pfizer Manufacturing Deutschland GmbH, Betriebsstatte Freiburg, respectively. All compounds were dissolved in saline solution (0.9% NaCl), but Aloperine also were dissolved in hydrochloric acid (5%). All compounds were injected intraperitoneally (i.p.) in an application volume of 0.1 ml/10 g body weight and were administered 15 min prior to testing for seven consecutive days from the 8th day.

2.3. CCI model surgery

Neuropathic pain was induced in experimental animals by CCI of the sciatic nerve which was performed as described method of Bennett and Xie [12]. Briefly, mice were anesthetized with sodium pentobarbital. Four ligatures (silk4-0) were tied loosely around proximal bifurcation part of the nerve with 1 mm spacing each ligature until a brisk twitch of the right hind limb was observed, respectively. In sham groups, an identical surgical procedure was performed, except that the sciatic nerve was not ligated [23].

2.4. Behavioral test

2.4.1. Von Frey filaments test

Mice were divided into seven groups: sham, CCI, CCI + Pregabalin (10 mg/kg), CCI + ALO 80 mg/kg, CCI + ALO 40 mg/kg, CCI + ALO 20 mg/kg, ALO 80 mg/kg group. Mechanical sensation of the hind paw as an index of mechano-allodynia was assessed as described method of Chaplan et al. [24]. Briefly, mice were placed in a Plexiglas box with a wire mesh grid that allowed their paws access to the von frey filaments. Von Frey filaments were applied to vertically stimulate the mid plantar surface of right hind paw until it bowed slightly. A modified version of the up–down paradigm was used. The 4.0 g filament was used as a cut-off. When clear a brisk withdrawal of the right hind paw were considered a positive reaction. Then, the force of the next filament was decreased or increased according to the response [23].

2.4.2. Cold-plate test

Mice were divided into seven groups: as Von Frey filaments test. Cold allodynia of the right hind paw was assessed using the cold plate as described method of Jasmin et al. [25]. Briefly, mice were placed in a Plexiglas box with metal plate that allowed access to the hind paws. The temperature of the metal plate was maintained at 4 ± 0.5 °C. Cold allodynia was sensitive to the reaction concerning either paw withdrawal. The total numbers of observation of hind paw withdrawal, licking or shocking on the operated side were recorded during the period of 5 min.

2.4.3. Radiant heat test

Mice were divided into seven groups: as Von Frey filaments test. Radiant heat hyperalgesia of the right hind paw was assessed by using PL-200 thermal sting apparatus as described method of Hargreaves et al. [26], for assessing the reactivity to noxious thermal stimuli. Briefly, mice were placed in a Plexiglas box and allowed to adapt themselves to the tested environment and temperature by 30 min. Then a radiant heat source was applied to vertically position under the plantar surface of the operated side hind paw. The cut-off time was 20 s to prevent tissue damage. The paw withdrawal latencies were recorded as the time interval between the start application of the heat beam and the first overt withdrawal appeared.

2.4.4. Paw pressure test

Mice were divided into seven groups: as Von Frey filaments test. Mechanical hyperalgesia of the hind paw was assessed by using YLS-3E electronic pressure apparatus as described by Randall and Selitto [27], for assessing sensitization to pressure stimulation. Briefly, mice were placed in a Plexiglas holder that allowed the right hind paw of mice to access to the pressure, until mice appeared nociceptive behavior reaction. Withdrawal of right hind paw was used to assess the mechanical nociceptive threshold that expressed in grams. The cut-off pressure was 450 g to prevent tissue damage.

2.4.5. Tail immersion test

Mice were divided into seven groups: as Von Frey filaments test. Spinal thermal hyperalgesia was assessed by the tail immersion test as described by Goyal et al. [28]. Briefly, mice were placed in a Plexiglas holder that allowed the mice tail to expose. The terminal part 3 cm of the mice tail was immersed in heat-noxious water (50 ± 0.5 °C), until the tail withdrawn above the water. The cut-off time was 15 s to prevent tissue damage.

2.4.6. T-AOC, T-SOD, GSH-PX and MDA estimation

All the mice were sacrificed by spinal dislocation on the 14th day after behavioral measurements. Then, the spinal cord (L4/5) was isolated from the body immediately. The spinal cord was homogenated with 0.9% saline using glass homogenate and centrifuged at 2500 r/min for 10 min. Supernatant of homogenate (10%,w/v) was employed for this test. Tissue protein concentration, T-AOC, GSH-PX concentration, T-SOD concentration and MDA levels were estimated by using protein quantitative kits, T-AOC kits,

GSH-PX kits, T-SOD kits and MDA kits from biological engineering research institute in Nanjing, respectively.

2.5. Western blot analysis

At the 14th day, mice were sacrificed by spinal dislocation. Then, the spinal cord (L4/5) was rapidly removed. Total proteins of the spinal cord were extracted by using protein extraction kits following the manufacturer's instructions. Protein concentration was estimated by using the KEYGEN Total Protein Extraction Kit. Protein was loaded for SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membrane with an electroblotting apparatus. The membranes were incubated with rabbit polyclonal antibody against NF-κB, TNF-α, IL-6, IL-1β and β-actin at 4 °C overnight and washed with PBST containing 20% Tween-20. The membranes were then incubated with the secondary antibodies goat of anti-rabbit. After washing with PBST, signals were visualized by Super Signal West Pico Chemiluminescent substrate (Pierce, USA) in a dark chamber and normalized to β-actin.

2.6. Data analysis

The analysis was performed using SPSS 16.0 software (Chicago, IL). All the results were expressed as mean ± standard; n refers to the numbers of experimental animals. Parametric values were analyzed by one-way analysis of variance (ANOVA) followed by the LSD post hoc test. For analyzing difference between two groups where equal variances of the data were not assumed, Tamhane's T2 test was used. In all statistical analyses, a value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. ALO alleviated mechanical allodynia

As shown in Fig. 1A, one day before surgery, the paw withdrawal threshold (PWT) value between groups showed no significant variation ($p > 0.05$). Seven days after surgery, mice subjected to CCI indicated significant mechanical allodynia as compared to the sham group ($p < 0.01$). However, administration of aloperine (80 mg/kg) exhibited no significant changes ($p > 0.05$). Compared with the CCI group, administration of aloperine (80 mg/kg, 40 mg/kg) and pregabalin (10 mg/kg) significant attenuated CCI induced decrease in PWT ($p < 0.01$), while there was no significant variation after giving aloperine 20 mg/kg ($p > 0.05$).

3.2. ALO alleviated cold allodynia

There were shown in Fig. 1B on the effects of aloperine on cold allodynia. One day before surgery, the counts of paw withdrawal value between groups showed no significant difference ($p > 0.05$). Seven days after surgery, the significant rising on the counts of paw withdrawal for mice subjected to CCI were observed ($p < 0.01$) but for mice accepted aloperine (80 mg/kg. i.p.) were not compared to the sham group.. When compared with the CCI group, the development of cold allodynia was significant attenuated by administration of aloperine (80 mg/kg, 40 mg/kg) ($p < 0.01$) and treatment of pregabalin also produced similar effects ($p < 0.01$). On the contrary, no protective effects for 20 mg/kg aloperine was showed ($p > 0.05$).

3.3. ALO alleviated thermal hyperalgesia

The effects of aloperine on thermal hyperalgesia in mice were shown in Fig. 1C. One day before surgery, there was no significant changes on paw withdrawal latency (PWL) among groups ($p > 0.05$). Seven days after surgery, mice subjected to CCI showed significant reduction in latency time ($p < 0.01$) compared to the sham group. Morever, mice accepted aloperine (80 mg/kg) exhibited no significant variation ($p > 0.05$). Compared with the CCI group, the development of thermal hyperalgesia was significant attenuated by administration of aloperine (80 mg/kg, 40 mg/kg) ($p < 0.01$) and treatment of pregabalin also produced similar effects, but not with the dose of 20 mg/kg ($p > 0.05$).

3.4. ALO alleviated mechanical hyperalgesia

There were shown in Fig. 1D, on the effects of aloperine on mechanical hyperalgesia in CCI mice. One day before operation, the mechanical nociceptive threshold showed no significant changes among groups ($p > 0.05$). Seven days after surgery, compared with the sham group, there were the significant decrease on mechanical nociceptive threshold for mice subjected to CCI were showed ($p < 0.01$), while for mice accepted aloperine (80 mg/kg) were not ($p > 0.05$). When compared with the CCI group, administration of aloperine (80 mg/kg, 40 mg/kg) and pregabalin (10 mg/kg) significant prevented CCI induced decrease in mechanical nociceptive threshold. On the contrary, lowest dose of 20 aloperine showed no significant variation ($p > 0.05$).

3.5. ALO alleviated spinal thermal hyperalgesia

The effects of aloperine on spinal thermal hyperalgesia in CCI mice were shown in Fig. 1E. One day before surgery, there was no significant variation in tail-curling latencies time between difference groups ($p > 0.05$). When compared with the sham group, seven days after surgery, mice subjected to CCI showed significant decrease in tailing latencies time ($p < 0.01$)and but administration of aloperine (80 mg/kg) was not ($p > 0.05$). Compared with the CCI group, administration of aloperine (80 mg/kg) remarkably reversed CCI induced decrease and treatment of pregabalin also produced similar effects ($p < 0.01$). On the contrary, there were no significant increase after giving aloperine 40 and 20 mg/kg ($p > 0.05$).

3.6. Anti-oxidation activity of ALO

After the 14th day of surgery, when compared with the sham group, mice of the CCI group generated significant decrease in T-AOC ($p < 0.01$), the levels of GSH-PX ($p < 0.05$), T-SOD levels ($p < 0.05$) and rise in MDA concentrations ($p < 0.01$). Further, administration of aloperine (80 mg/kg. i.p.) showed no statistical difference ($p > 0.05$). Compared to the CCI group, the significant reduction in T-AOC, GSH-PX levels, T-SOD levels and rise in MDA concentrations for administration of aloperine (80 mg/kg) were observed. Treatment of pregabalin also produced similar effects. (Table 1)

3.7. NF-κB, TNF-α, IL-6 and IL-1β expression

The protein expression levels of NF-κB, TNF-α, IL-6 and IL-1β in CCI group significantly increased compared with those of the sham group ($p < 0.01$). When compared with the CCI group, the protein expression levels of NF-κB, TNF-α, IL-6 and IL-1β markedly decreased in ALO 80 mg/kg group ($p < 0.01$). (Fig. 2)

4. Discussion

At present, conventional medicines such as opioids and nonsteroidal anti-inflammatory drugs, tricyclic anti-depressants and anti-convulsants were unable to control this pain symptom effectively

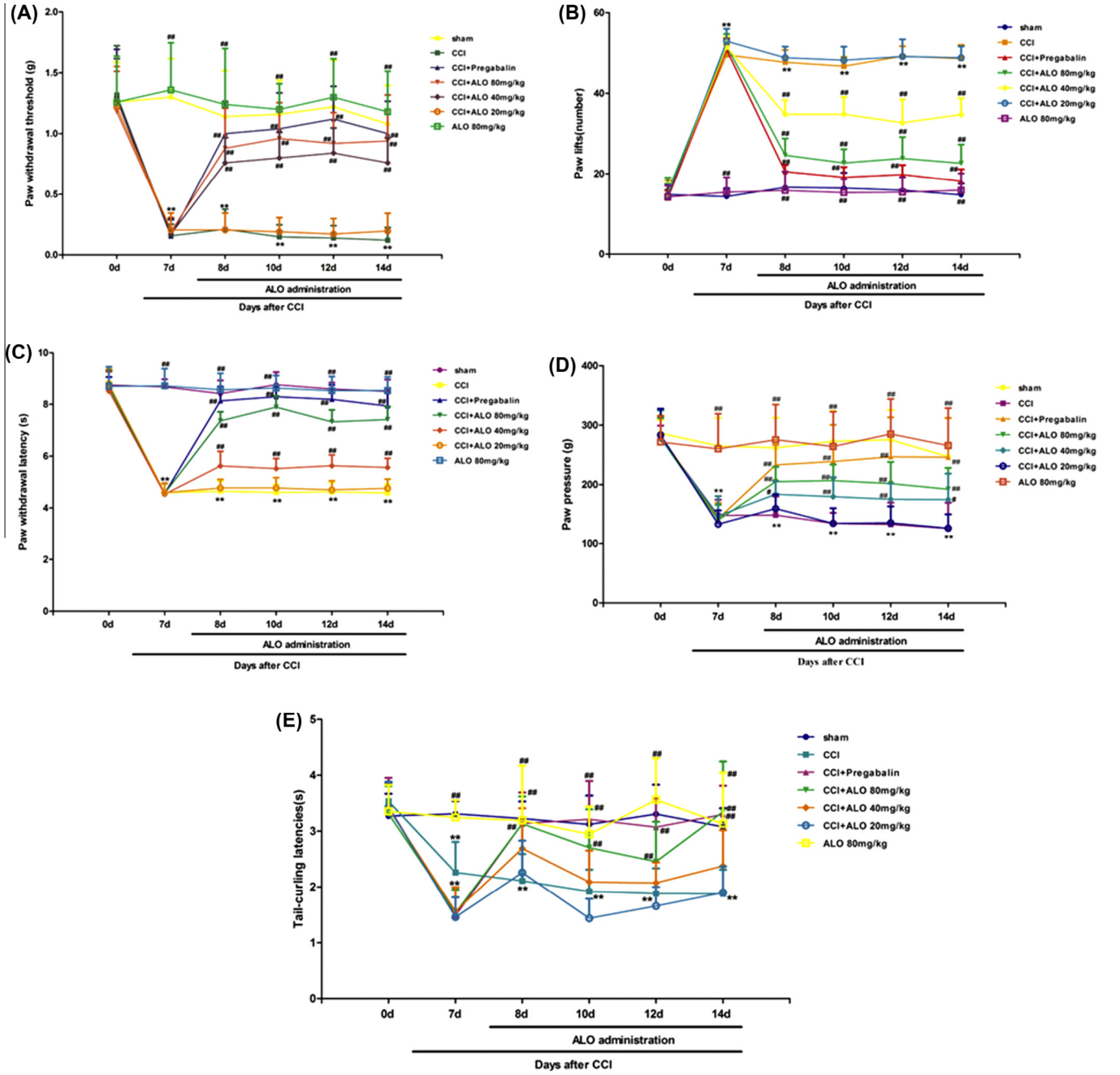

Fig. 1. (A–E) Effects of ALO on mechanical withdrawal threshold, cold withdrawal counts, thermal withdrawal latency, mechanical withdrawal threshold and thermal tail-curling latency in neuropathic pain mice. Data are expressed as mean ± S.D. each group consist of ten ($n = 10$). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the sham group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the CCI group.

[29,30]. Also, studies have showed that these drugs exhibited a wide spectrum of adverse effects which limited their full clinical exploitation in treatment of painful neuropathy [31–33]. Therefore, novel, efficacious and safe analgesic agents for neuropathic pain are urgently needed. Traditional Chinese medicine has shown intriguing potential due to its abundant resources, multitargeted mechanisms of activity, few side effects, and no drug resistance. Aloperine is one alkaloid extracted from *S. alopecuroides* L. (Leguminosae) which is a type of traditional Chinese herbal and mainly existed in leaves. In addition, *S. alopecuroides* L. (Leguminosae) are commonly used as analgesic [8]. At the same time, in preliminary experiment, we used behavior tests to evaluate the anti- allodynia and anti-hyperalgesia effects of ALO on neuropathic pain and filtrated optimal dosage rang. The results showed that aloperine attenuated allodynia and hyperalgesia induced by CCI and the best dosage rang was 80-20 mg/kg. Therefore, the present study was designed to evaluate antinociceptive potential of systemically administered aloperine in neuropathic pain mice induced by CCI. In the present study, the results of the behavior tests showed that aloperine had antinociceptive effects on mechanical allodynia and hyperalgesia, thermal hyperalgesia and cold allodynia induced neuropathic pain.

Several studies have evidenced that ROS played an important role in neuropathic pain [18,19]. In animal experiments, intrathecal injection of superoxide donor could enhance neuropathic pain and induced hyperalgesia [34]. In addition, studies also have indicated that intraperitoneal administration of Phenyl N-tert-butylnitrone, which is a free radical scavenger could reduce

Table 1
The levels of T-AOC, GSH-PX, T-SOD and MDA in the spinal cord of neuropathic pain mice.

Groups	Dose mg/kg	T-AOC U/mgprot	GSH-PX U/mgprot	T-SOD U/mgprot	MDA nmol/mgprot
Sham	–	0.114 ± 0.024	355.77 ± 79.66	28.72 ± 7.03	3.34 ± 0.54
CCI	–	0.046 ± 0.011**	272.10 ± 60.69*	18.90 ± 1.92*	4.84 ± 0.58**
CCI + Pre	10	0.079 ± 0.029$^{*,\#}$	336.59 ± 81.06$^{\#\#}$	22.27 ± 3.72$^{\#}$	3.47 ± 0.23$^{\#\#}$
CCI + ALO	80	0.078 ± 0.027$^{**,\#}$	315.48 ± 53.69$^{\#\#}$	21.95 ± 3.91$^{\#}$	3.57 ± 0.69$^{\#\#}$
CCI + ALO	40	0.054 ± 0.015$^{**,\#}$	306.63 ± 82.29$^{\#}$	21.26 ± 4.49$^{\#}$	3.71 ± 1.09$^{\#}$
CCI + ALO	20	0.047 ± 0.007**	278.00 ± 71.96*	18.90 ± 5.06*	4.06 ± 0.83**
ALO	80	0.104 ± 0.029$^{\#\#}$	348.56 ± 66.28$^{\#}$	29.39 ± 6.88$^{\#}$	3.19 ± 0.69$^{\#\#}$

Data are expressed as mean ± S.D. each group consist of six (n = 6). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the sham group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the CCI group.

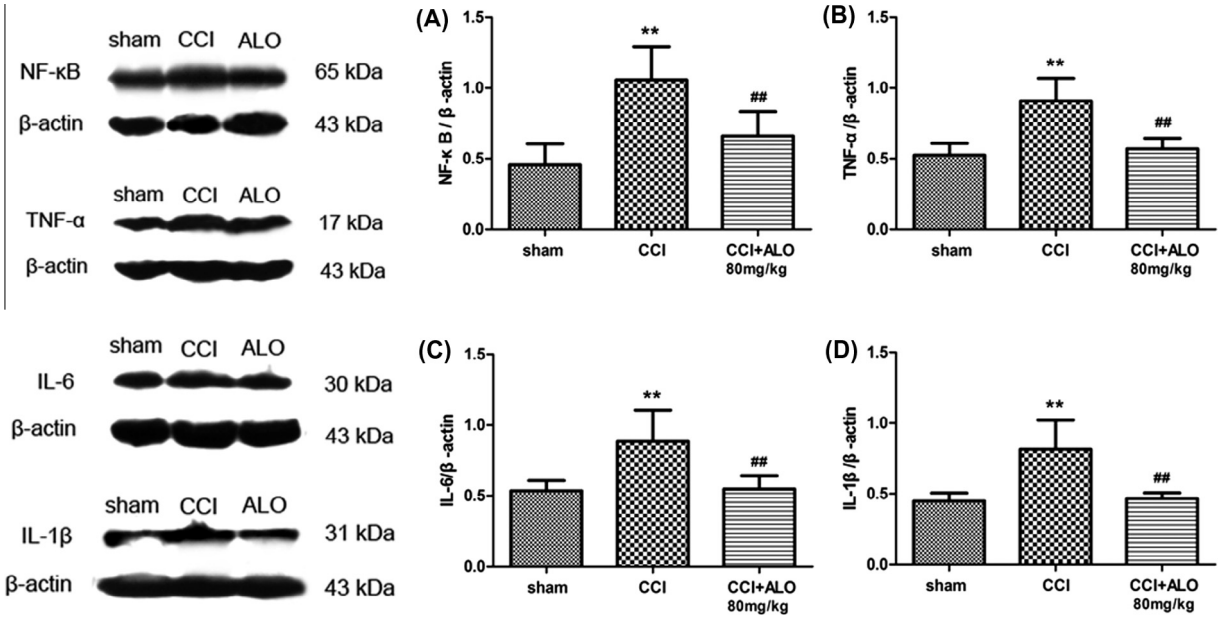

Fig. 2. ALO down-regulated the expression levels of NF-κB, TNF-α, IL-6 and IL-1βin the spinal cord of neuropathic pain mice. Figure A to D showed the expression levels of NF-κB, TNF-α, IL-6 and IL-1β target proteins in the spinal cord of neuropathic pain mice by using Western blot analysis. Data are expressed as mean ± S.D. each group consist of six (n = 6). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the sham group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the CCI group.

mechanical allodynia in chemotherapy-induced neuropathic pain in rats [35]. In the present study, in the terms of results of T-AOC, GSH-PX, T-SOD and MDA measurements, this prompted that aloperine alleviated allodynia and hyperalgesia induced by neuropathic pain due to anti-oxidation activity.

Recently, studies have indicated that NF-κB and its downstream pro-inflammatory cytokines played a vital role in neuropathic pain [18]. NF-κB is a pleiotropic transcriptional factor which located in cytoplasm and regulated the genes expression of many factors associated with pain (TNF-α, IL-6, et al.) [36,37]. Studies have showed that intrathecal administration of PDTC, an NF-κB inhibitor, reversed mechanical allodynia in neuropathic pain condition [38]. In addition, after nerve damage, endoneurial administration of NF-κB decoys remarkable attenuated thermal hyperalgesia [39].

TNF-α is regarded as the prototypic pro-inflammatory cytokine, which played a vital role in triggering the cascade of activation of other cytokines in the inflammatory responses. Studies have shown that endoneurial administration of TNF-α could induce the mechanical allodynia and thermal hyperalgesia in the rat CCI model [40]. On the contrary, interdiction of TNF-α produced the opposite effects [41,42].

IL-1β and IL-6 are also pro-inflammatory cytokines involved in neuropathic pain [43]. Studies have indicated that intrathecal administration of IL-1β could produce hyperalgesia and allodynia [44,45]. However, intrathecal injection of IL-1β receptor antagonist markedly attenuated allodynia and hyperalgesia in neuropathic pain models of rats [46]. In addition, studies have also revealed that intrathecal injection of an IL-6 neutralizing antibody significantly reduced nerve injury-induced mechanical allodynia [47].

In the present study, significantly up-regulation of NF-κB, TNF-α, IL-6, IL-1β protein expression levels in the dorsal spinal cord after CCI were observed, along with remarkable reduction of those protein expression levels for aloperine at the dose of 80 mg/kg, which was consistent with the above studies. Therefore, we concluded that aloperine against neuropathic pain induced by CCI was related to the pathway of NF-κB and its downstream pro-inflammatory cytokines.

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