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Sodium Aescinate Alleviates Neuropathic Pain in Rats by Suppressing the TLR4/NF KB Pathway Activation after Paclitaxel Chemotherapy

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Abstract

Background: Emerging evidence suggests that chemotherapy-induced peripheral neuropathy (CIPN) is a significant side effect of chemotherapeutic drugs. Many experiments have proved that sodium aescinate (SA) has definite pharmacological effects such as anti-infection, anti-exudation, anti-edema, anti-tumor as well as neuroprotection, and the drug side effects are mild. However, no study has explored whether SA is involved in the analgesic effect of paclitaxel (PAC) induced neuropathic pain in rats. **Methods:** Rats were given an intraperitoneal injection of PAC (2.5 mg/Kg intraperitoneally on days 1, 3, 5, and 7), while SA 25 mg/kg intraperitoneally was administered daily for 14 consecutive days. The mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) of rats were examined on experimental days 3, 5, 7, 11, 14. All rats were sacrificed on day 15 of the experiment, and L4-6 spinal cords were removed. Subsequently, immunohistochemistry, HE staining, ELISA, RT-qPCR, Western blotting were applied to evaluate cytoskeletal protein expression (NF-L and NF-M), spinal nerve structural integrity, proinflammatory factor contents (TNF-α, IL-1β, and IL-6), and protein content of the TLR4/NF-κB pathway, respectively. **Results:** After the rats developed PAC induced pain behaviors, multiple injections of SA rendered the rats with elevated MWT and TWL values, decreased expression of NF-L and NF-M in the spinal cord, materially downregulated content of proinflammatory factors, and reduced amounts of TLR4 and p-NF-κB protein levels. **Conclusions:** The results of the present study preliminarily indicate that SA has an analgesic effect on rats with CIPN induced by PAC injection, and the mechanism may be related to blocking the TLR4/NF-κB signaling pathway, inhibiting the expression of proinflammatory factors, and alleviating cytoskeletal disorders.

Keywords

Chemotherapy-Induced Peripheral Neuropathy, Sodium Aescinate, The TLR4/NF-κB Pathway, Paclitaxel

1. Introduction

Cancer seriously endangers human health, and its incidence is increasing year by year. There are currently approximately 7 million new cancer patients occurring annually worldwide according to the World Health Organization [1]. Chemotherapy is one of the current important modalities for treating tumors, and although it can prolong the survival time of patients, it is accompanied by some distressing side effects [2]. Among them, chemotherapy-induced peripheral neuropathy (CIPN) is one of the common side effects in cancer patients and refers to the use of a specific class of chemotherapeutic drugs (phytoalkaloids, paclitaxels, platinum, etc.) resulting in the impairment of sensory, motor, autonomic nerve conduction, of which the symptom of sensory nerve damage is the most common [3]. The current clinical use of platinum, paclitaxel (PAC), and vincristine-based chemotherapeutics has been associated with a higher incidence of CIPN [4]. Statistically, acute onset of CIPN occurs in 65% - 96% and conversion to chronic CIPN occurs in 40% - 93% following oxaliplatin use [5]. The incidence of CIPN following cisplatin use is 12% - 85% [6]. Vincristine exposure occurs in 20%. The highest incidence of CIPN is seen with paclitaxels, ranging from 61% - 92%, and 30% of patients continue to endure the pain of CIPN 6 months after completion of chemotherapy [7]. At present, the lack of information related to the treatment of CIPN makes the patient's symptoms not effectively controlled, which in turn affects the progress or even the termination of chemotherapy. CIPN persists even after the completion of chemotherapy, leaving patients facing physical and functional challenges, experiencing social difficulties, emotions, sleep disturbances, etc., and severely affecting quality of life. Therefore, the development of effective drugs against CINP is a very urgent thing for cancer chemotherapy patients.

Aescinate is a natural mixture of triterpenoid saponins extracted from the seeds of *S. aestivum* after mature drying, and its major components are aescinate A, aescinate B, aescinate C and aescinate D [8]. In clinic, it is mostly applied in the form of tablets, gels, lyophilized powder of sodium aescinate (SA), and so on, and the oral form of SA tablets is most widely used [9]. SA has high solubility in water and good stability. A large number of experiments have proved that SA has definite pharmacological effects such as anti-infection, anti-exudation, anti-edema, anti-tumor as well as neuroprotection, and the drug side effects are mild [9] [10] [11]. At present, SA is widely used in the clinic for the treatment of diseases such as intracerebral hemorrhage, edema due to exudation after tissue

trauma, acute facial neuritis, lumbar disc herniation, herpes zoster, and venous thrombosis [12] [13]. In recent years, the study of SA in analgesia has also received industry attention. Li *et al.* [14], in their study of formalin induced pain, found that pain in rats was relieved by intrathecal administration of SA. A recent study found that SA significantly reduced the duration of licking, the number of flinches and increased paw edema in rats with neuropathic pain induced by chronic constriction injury of the sciatic nerve, showing great therapeutic effects on inflammatory pain responses [15]. However, no study has yet confirmed whether SA similarly has a mitigating effect on CINP.

Taken together, we speculate that SA has the potential to be an effective agent for the treatment of CINP. Since SA has made preliminary attempts and achieved certain effects in the direction of neuropathic pain, it will provide more substantial experimental evidence for its application in the field of neuropathic pain. In addition, since CINP incidence is highest after the use of taxanes, PAC induced peripheral neuropathic pain was adopted as a subsequent CINP research model in this study. Taking this as a hint, this study hopes to explore its alleviating effect on PAC induced neuropathic pain in rats by establishing the administration of SA to CINP rats, and to make a preliminary study on the mechanism of drug action.

2. Materials and Methods

2.1. Animal Experiment Grouping

Thirty-six SPF grade male SD rats aged 8 - 10 weeks with a body mass of 250 - 300 g were purchased from the Animal Experiment Center of Xi'an Jiaotong University. The rats were placed in animal facilities recognized by the International Laboratory and Nursing Evaluation and Certification Association, and were fed adaptively for a week using standard bedding during a 12 - 12 hour light and dark cycle at 24˚C, with free access to food and water. The rats were randomly divided into four groups according to the experimental design, including control, paclitaxel (PAC), and PAC + SA, and with 12 rats in each group. Animal experiments were approved and supervised by the Animal Ethics Committee of Shaanxi Provincial People's Hospital.

2.2. Establishment of the CINP Model

For the paclitaxel group, rats were administered PAC intraperitoneally on days 1, 3, 5, and 7 at the start of the experiment at a single dose of 2.5 mg/Kg for a total of four injections, and the final cumulative dose of paclitaxel was 10 mg/Kg. The mechanical withdrawal thresholds (MWT) and thermal withdrawal latency (TWL) were significantly reduced on the 3rd day, indicating the successful construction of CINP model. For the PAC + SA group, in addition to the intraperitoneal injection of PAC (2.5 mg/Kg intraperitoneally on days 1, 3, 5, and 7), SA 25 mg/Kg intraperitoneally was administered daily for 14 consecutive days after the start of the experiment. The same dose of saline was injected intraperito-

neally on days 1, 3, 5, and 7 in the control group. The rats were sacrificed on the 15th day of the experiment, and the spinal cords of the L4-6 group were taken for subsequent studies.

2.3. Behavioral Detection of Neuralgia in Rats

Behavioral tests were performed using von Frey filaments and thermal stimuli on rats before (day 0) and on days 3, 5, 7, 9, and 11 after the start of the experiment. Mechanical stimulus paw withdrawal thresholds (MWT) determined from Von Frey filament experiments and thermal withdrawal latency (TWL) determined from thermal stimuli experiments were used as behavioral measures. TWL: A single rat was placed in a clear glass box and allowed to acclimate in a quiet environment for 30 min. A thermal pain stimulus instrument was used to irradiate the bottom of the left hind foot of the rats, respectively, and the irradiation was stopped when the rats showed a characteristic licking or paw lifting response. Subsequently, the latency time (s), *i.e.*, TWL, of the rats in response to thermal stimuli was recorded. Each rat was tested 5 times with 5-min intervals between each session. MWT: Rats were acclimated for 30 min in a plastic box, and the right hind foot of the rats was stimulated with a Von Frey probe representing different pressures (minimum 0.4 g, maximum 26.0 g), each lasting 5 s. A rapid paw withdrawal was defined as a positive response when the rats presented, at which time the stimulus pressure was recorded. Subsequently, the MWT (g) was calculated for each rat, and each rat was tested five times with an inter stimulus interval greater than 2 min and averaged.

2.4. Immunohistochemical Staining

Spinal cord sections from rats in each group were deparaffinized, hydrated, antigen retrieval, serum blocking, NF-L (ab223343, Abcam, UK) and NF-M (ab7794, Abcam, UK) primary antibody and corresponding secondary antibodies were incubated, washed, DAB developed, hematoxylin counterstained, and the mounting of sections was performed sequentially.

2.5. HE Staining

The spinal cord tissues of rats in each group were preserved and fixed using 4% paraformaldehyde at 4˚C for one week. After tissue paraffin embedding and wax block sectioning, hematoxylin, and eosin (HE) staining (Thermo-fisher, USA) was used according to the manufacturer's requirements. Finally, slides were blocked after dehydration with ethanol, and then the staining was observed under a microscope.

2.6. Enzyme Linked Immunosorbent Assay (ELISA)

The levels of tumor necrosis factor-α (TNF-α, KALANG, USA), interleukin-1β (IL-1β, KALANG, USA), and interleukin-6 (IL-6, KALANG, USA) were measured by enzyme-linked immunosorbent assay, and the experimental procedures

were strictly performed according to the kit instructions.

2.7. RT-qPCR

The spinal cords of rats with different treatments in each group were collected, and total RNA was extracted. The cDNA was then used as template for mRNA (TNF-α, IL-1β, IL-6) amplification on a Bio-Rad CFX90 Real time PCR. GAPDH was used as an internal reference, and the relative expression levels were calculated by using the $2^{-\Delta\Delta CT}$ method.

2.8. Western Blotting

Each group of rat spinal cords was washed 2 times with cold PBS and lysed according to the application in RIPA lysis buffer (Roche Diagnostics, Basel, Switzerland) filled with protease inhibitors. The BCA method was used to perform protein quantification, followed by SDS-PAGE electrophoresis, in which 50 μg of sample was added to each well and the constant pressure was 80 V. A constant voltage of 120 V was set after 30 min for 75 - 90 min. A final constant voltage of 20 V was applied semi dry electric rotation for 1 h, and the proteins were transferred to a PVDF membrane. After blocking the membrane with 5% nonfat dry milk in PBST for 1 h, the primary antibody was incubated overnight at 4˚C. The following primary antibodies: rabbit anti-β-actin (ab8227, Abcam, UK), rabbit anti-TLR4 (ab13556, Abcam, UK), rabbit anti-NF-κB p65 (ab32536, Abcam, UK), rabbit anti-p-NF-κB p65 (ab76302, Abcam, UK). After finally washing the membrane with PBST, the membrane was incubated with secondary antibody at room temperature for 1 h, and the membrane was washed again for ECL (Pierce, Rockford, IL, USA). β-actin served as an endogenous contrast.

2.9. Statistical Analysis

Data are processed using SPSS 22.0 statistical software and presented as the mean ± SEM of results from at least three independent experiments. Differences among more than two groups in the above assays were estimated using one-way ANOVA, with $P < 0.05$ considered significant

3. Results

3.1. Analgesic Effects of Multiple Injections of SA on Established Paclitaxel Induced Neuropathic Pain

Firstly, the rats were found to have normal weight gain after SA treatment was administered in the PAC induced CINP model (**Figure 1(a)**). Subsequently, it was shown 3 days after the start of the experiment that the values of both MWT and TWL were conspicuously decreased in the paclitaxel group compared with the control group, suggesting that the simulated CINP model was constructed successfully. In addition, the values of both MWT and TWL in rats after administration of multiple injections of SA were elevated compared with the PAC group (**Figure 1(b)**, **Figure 1(c)**). These above illustrate that multiple injections of SA have analgesic effects on rats with paclitaxel induced neuropathic pain.

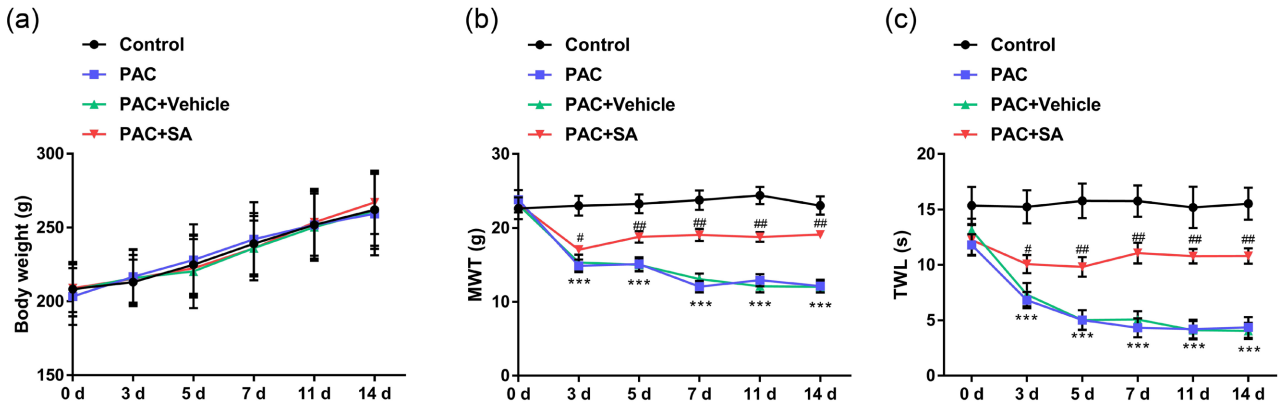

Figure 1. Analgesic effects of multiple injections of SA on established paclitaxel induced neuropathic pain. (a) The time courses of body weight in paclitaxel (PAC)-induced neuropathic pain in rats following SA treatment. (b) The time course of paclitaxel (PAC)-induced mechanical withdrawal thresholds (MWT) for neuropathic pain in rats following SA treatment. (c) The time course of paclitaxel (PAC)-induced thermal withdrawal latency (TWL) for neuropathic pain in rats following SA treatment. Data were presented as mean ± SEM. N = 10. ***$P < 0.001$ vs Control group; #$P < 0.05$, ##$P < 0.01$ vs PAC group.

3.2. SA Ameliorates Paclitaxel Induced Neuropathic Pain by Inhibiting Spinal Neurofilament Protein Expression in Rats

Next, the expression of the neurofilament proteins NF-L and NF-M in the rat spinal cord was detected using immunohistochemistry. Abnormal accumulation of neurofilament proteins in the neuronal soma or axon is known to be a hallmark of motor neuron disease and is also considered a marker of neuronal axonal degradation. The results of this study display that NF-L and NF-M are barely expressed in the spinal cord of control rats, and NF-L and NF-M expression is preeminently elevated in the PAC group (**Figure 2(a)**, **Figure 2(b)**), suggesting that axonal damage resulting from PAC may contribute to the development of pain, whereas SA injection decreases NF-L and NF-M expression and alleviates neuropathic pain induced by PAC.

3.3. SA Suppresses Paclitaxel Induced Neuroinflammation in the Rat Spinal Cord

Inflammatory factors TNF-α, IL-1β, and IL-6 have been shown to contribute to neuropathic pain, and these receptor antagonists are effective in alleviating hyperalgesia in animal models of neuropathic pain. According to this, the contents of pro-inflammatory factors of mice in each group were further analyzed in this study. RT-qPCR and ELISA results suggested that neuroinflammation was activated in the rat spinal cord after PAC repeated injections, as indicated by a significant increase in the mRNA and protein content of the proinflammatory factors. However, the contents of the above proinflammatory factors in the spinal cords of rats treated with SA repeated injection were materially downregulated (**Figure 3(a)**, **Figure 3(b)**). Further, it was observed from HE staining of rat spinal cords that the control group had an intact spinal nerve structure and no inflammatory cell infiltration, whereas the PAC Group had obvious inflamma-

tory cell invasion, which was improved by SA injection (Figure 3(c)). Taken together, these results demonstrated that SA inhibits paclitaxel induced neuroinflammation in the rat spinal cord.

3.4. SA Inhibited Paclitaxel Mediated Activation of the TLR4/NF-κB Pathway

Previous studies have proposed that activation of the TLR4/NF-κB pathway is also involved in the development of neuropathic pain resulting from PAC. The results of this study display that TLR4 and p-NF-κB protein expression, but not NF-κB total protein, were substantially upregulated in the spinal cords of rats with repeated PAC injections, whereas they were suppressed after SA injection treatment (Figure 4). These results suggest that SA may be able to target the TLR4/NF-κB pathway to ameliorate PAC-mediated neuropathic pain in the rat spinal cord by inhibiting its activation.

4. Discussion

Because of the poor selectivity of chemotherapeutic drugs, it is also inevitable to damage normal human cells while killing cancer cells, resulting in the adverse effects of drugs, among which pain is a common type of adverse effect when chemotherapy is applied [16]. At present, CIPN has become an important reason that seriously affects the survival quality of cancer patients, and how to effectively avoid or slow the occurrence of CIPN has become an urgent problem during chemotherapy [17]. Therefore, to effectively reduce pain when cancer patients are treated with chemotherapy, the rational use of analgesic drugs has become the most important clinical means for the treatment of cancer pain at

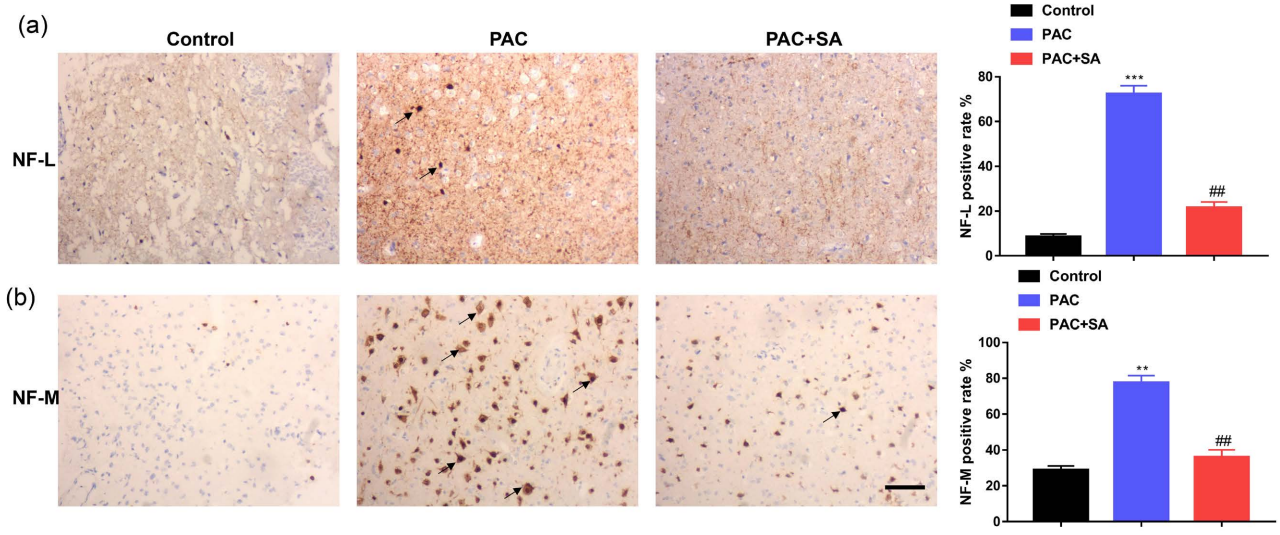

Figure 2. SA ameliorates paclitaxel induced neuropathic pain by inhibiting spinal neurofilament protein expression in rats. (a) Immunohistochemistry detection of the expression of neurofilament proteins NF-L in rat spinal cords after SA treatment. (b) Immunohistochemistry detection of the expression of neurofilament proteins NF-M in rat spinal cords after SA treatment. Scale bar: 50 μm. Data were presented as mean ± SEM. N = 10. $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control group; $\#\#P < 0.01$ vs PAC group.

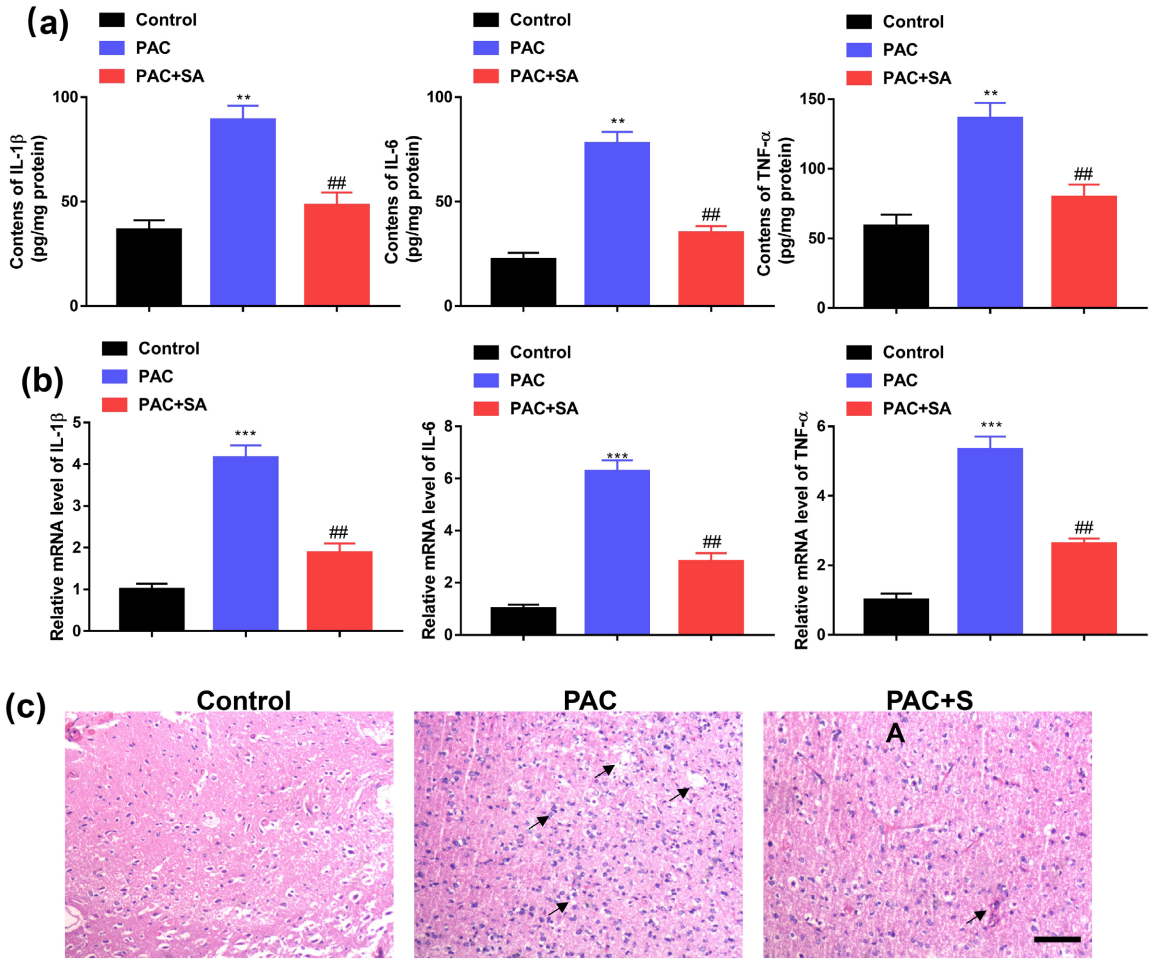

Figure 3. SA suppresses paclitaxel induced neuroinflammation in the rat spinal cord. (a) ELISA assessment of the protein contents of proinflammatory factors TNF-α, IL-1β, and IL-6 in rat spinal cords after SA treatment. (b) RT-qPCR estimate of the mRNA expression of proinflammatory factors TNF-α, IL-1β, and IL-6 in rat spinal cords after SA treatment. (c) H&E staining of spinal cords from rats in each group, with red arrows pointing to disrupted neural structures. Scale bar: 50 μm. Data were presented as mean ± SEM. N = 10. $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control group; $^{\#\#}P < 0.01$ vs PAC group.

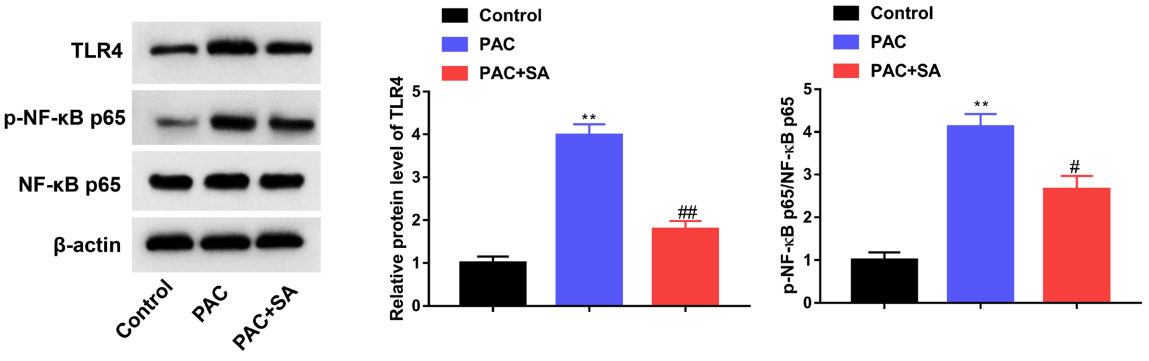

Figure 4. SA inhibited paclitaxel mediated activation of the TLR4/NF-κB pathway. Western blotting assessment of the protein levels of the TLR4/NF-κB pathway related proteins TLR4, p-NF-κB, and NF-κB in rat spinal cords after SA treatment. Data were presented as mean ± SEM. N = 10. $^{**}P < 0.01$ vs Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs PAC group.

present. In this study, we established a CIPN rat model by referring to the dosage and manner of PAC administration commonly used in the literature, and the results showed that intraperitoneal administration of PAC at 2.5 mg/Kg every 2 days for four consecutive administrations was able to effectively induce mechanical pain behaviors in rats. However, MWT, TWL and other behaviors of rats were improved after administration of SA, suggesting that the mechanism of SA application may be associated with neuropathic pain. Previous studies have also discovered that SA is associated with multiple types of neuropathic pain. Yao *et al.* [18] demonstrated that SA can attenuate ischemia-reperfusion injury of nervous tissue due to nerve ligation and play a promoting role in the recovery of neurological function, thereby alleviating neuralgia. In addition, SA has also been confirmed to be able to exert antitumor effects in a variety of cancers [19] [20]. Subsequently, the present study further found that PAC induced neuropathic pain may be associated with its mediated disturbance of neurofilament proteins in the spinal cord. Neurofilament proteins are the major structural units of the cytoskeleton specifically expressed in neurons and their axons in the central and peripheral nervous systems [21]. Neurofilament proteins consist of light (NF-L), medium (NF-M), heavy (NF-H) proteins and α-catenin. Abnormal accumulation of neurofilament proteins in the neuronal soma or axon is a hallmark of motor neuron disease and is also considered a marker of neuronal axonal degradation [22]. The results of this study suggest that PAC treatment of rats significantly elevated NF-L, NF-M expression in spinal cord tissue, suggesting that axonal damage resulting from PAC may contribute to the development of pain, whereas administration of SA treatment reduced NF-L, NF-M expression and alleviated neuropathic pain induced by PAC. The latest studies have also shown that the levels of NF-L are markedly elevated in the serum of patients with chronic pain following rehabilitation from COVID-19, and NF-L may serve as a potential biomarker for persistent neuropathic pain on COVID-19 [23]. Furthermore, Yang *et al.* [24] found that the levels of NF-L polypeptide were elevated in a pain model constructed by spinal nerve ligation in rats, and miR-7a could block transcriptional signaling pathway activators by inhibiting NF-L polypeptide and then ameliorate neuropathic pain.

Cytokines are proteins that regulate systemic immune responses. Cytokines involved in innate immune signaling have also been identified as key players driving the pathophysiology of CIPN [25]. Cytokines are released not only by immune cells but also by glia and neurons [26]. They can directly or indirectly act on primary afferent fibers, dorsal root ganglia, and spinal dorsal horn neurons and are involved in the development and progression of inflammatory, cancerous pain, and morphine tolerance [27]. The production and release of proinflammatory factors, which can be conspicuously increased by chemotherapeutic drugs, is considered to be one of the main mechanisms regulating CIPN [28]. The results of this study show that the levels of the proinflammatory cytokines are remarkably upregulated in the PAC administered group. However, the con-

tents of the above proinflammatory factors in the spinal cords of rats treated with SA repeated injection were materially downregulated.

TLR4 in the spinal cord plays an important role in models of neuropathic pain and nerve injury [29]. Studies have shown that chemotherapeutic agents are responsible for the activation of TLR4 leading to increased proinflammatory cytokine expression in the peripheral and central nervous systems [30]. The activation of TLR4 is ultimately manifested in the activation of nuclear factor κB (NF-κB), which promotes inflammatory factor and chemokine synthesis and secretion, initiating acquired immune responses [31]. Chang *et al.* [32] showed that kaempferol treatment strikingly reduced neuropathic pain and proinflammatory cytokine production, and attenuated TLR4/NF-κB pathway activation. Wang *et al.* [33] suggested that dexmedetomidine could alleviate neuropathic pain and reduce inflammatory responses by preventing the activation of TLR4/NF-κB p65 pathway. These above suggest that inhibiting the activation of TLR4/NF-κB pathway may be an effective drug target for alleviating neuropathic pain. However, whether the TLR4/NF-κB p65 signaling pathway is expressed and functions in the PAC induced CIPN model remains unknown. The results of the present study are consistent with previous findings and that SA can restrain the TLR4/NF-κB p65 pathway to ameliorate PAC mediated neuropathic pain in the rat spinal cord.

5. Conclusion

The results of the present study preliminarily indicate that SA has an analgesic effect on rats with CIPN induced by PAC injection, and the mechanism may be related to blocking the TLR4/NF-κB signaling pathway, inhibiting the expression of proinflammatory factors, and alleviating cytoskeletal disorders. This study provides an initial exploration of the therapeutic effects of SA on PAC induced CIPN. However, the experimental content is relatively insufficient, and more experimental data are needed next to do further validation and refine the relevant mechanism of action studies of the drug.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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