Sodium aescinate ameliorates chronic neuropathic pain in mice via suppressing JNK/p-38-mediated microglia activation

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Abstract

A study confirmed that sodium aescinate (SA), a traditional Chinese medicine extracted from the dried ripe fruits of the aescin plant chestnut, can effectively relieve bone cancer pain, but its role in neuropathic pain (NP) remains confused. This study aimed to investigate whether SA has a protective effect on NP and its underlying mechanisms. Thirty mice were randomly divided into three groups (n = 10 per group): sham + vehicle, chronic contraction injury (CCI) + vehicle, CCI + SA. SA (40 μg/L, intrathecal injection) was administered once daily for 5 consecutive days starting on day 7 after surgery. The mechanical withdrawal thresholds (paw withdraw threshold, PWT) of the contralateral and ipsilateral paws of mice in each group were subsequently detected daily. The results displayed that repeated SA treatment could prominently increase the reduction of PWT induced by CCI in the ipsilateral paw of mice. Downregulation of p- c-Jun N-terminal kinase (JNK) and p-p38 protein levels and reduction of microglial activation marker Iba-1-positive ratio, M1/M2 ratio of microglia, and proinflammatory factors, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, in the spinal cords of CCI-mice was observed after intrathecal SA. The above data illustrated that SA might suppress the activation of microglia and neuroinflammation by selectively inhibiting the JNK/p38 signaling pathway, which in turn alleviated CCI-induced NP in mice.

1 Introduction

Neuropathic pain (NP) is a severe chronic pain condition defined by the International Association for the study of pain as pain caused by a lesion or disease in the somatosensory nervous system, which imposes a heavy physical, psychological, and economic burden on patients(Baron, Binder,Wasner 2010). Epidemiological observations indicate that approximately 6.9% − 10% of the global population has or has ever suffered from NP(Finnerup, Kuner,Jensen 2021). According to statistics, the annual cost of health care and productivity loss due to NP is up to $600 billion in the United States(Mbrah, Nunes, Hume et al. 2022). Clinical studies have shown that NP is not a specific disease, but is a clinical symptom caused by a variety of different diseases and lesions, injury at the level of the peripheral or central nervous system, leading to peripheral or central NP, respectively(Gurba, Chaudhry,Haroutounian 2022). It is characterized by spontaneous pain, mechanical hyperalgesia, and touch pain, which is often manifested as burning, stabbing or electric shock like pain and can be caused by sciatic nerve compression, cancer, infection, autoimmune diseases, trauma, and diabetes, among others(Gilron, Baron,Jensen 2015). NP is caused by damage to the somatosensory system, progresses slowly, and depends on the extent of damage to both the peripheral and central nervous systems. It is affected not only by the persistence of peripheral nociceptive signals, but also by pathological functional changes in the nervous system, shifting the state of the excitation inhibition balance of neuronal networks toward excitability and thereby reducing the inhibition of neuronal firing activity, and changes in the function of glial cells(Tsuda 2016). Thus, it affects the processing process of pain signals, which decreases the pain threshold and finally leads to the formation of peripheral sensitization and central sensitization.

Previous studies have shown significant functional, structural, and molecular changes in glial cells in animal models of NP following nerve injury(Inoue and Tsuda 2018). Peripheral nerve injury leads to significant activation of glial cells, including Schwann cells in nerves, satellite glial cells in dorsal root ganglia, microglia and astrocytes in the spinal cord and brain, among others. The proportion of glial cells in the central nervous system (CNS) is approximately 70% and includes microglia, astrocytes, and oligodendrocytes(Yi, Liu, Liu et al. 2021). There is increasing evidence that many pathological processes within the CNS are mediated by microglia, as in alzheimer's disease, parkinson's disease, psychiatric disorders as well as NP(Hansen, Hanson,Sheng 2018; Ho 2019; Kim, Choi,Yoon 2020; Kong, Li, Deng et al. 2022). Kohno *et al.*(Kohno, Shirasaka, Yoshihara et al. 2022) confirmed that suppression of the molecular function or activation of microglia inhibited the abnormal excitability and NP of dorsal horn neurons. Thus, it is suggested that spinal microglia are central players in NP mechanisms and may be a potential pharmacotherapeutic target for the treatment of chronic pain states. However, the number of people who achieve significant relief from their painful condition clinically with pharmacotherapy still falls short of half the total number of affected people. Moreover, it is characterized by the chronicity of the disease course, poor treatment efficacy, many side effects and high burden of medical expenditure, which seriously affect the quality of life of patients. Therefore, finding more effective, less adverse effects and cheaper drugs will be one of the important directions for future research on NP. Traditional Chinese medicine (TCM) displays unique advantages due to its few side effects, multiple mechanism targets and rich sources.

Sodium aescinate (SA), a multi-ester bond containing triterpenoid soap extracted from the dried ripe fruits of the aescirrhizal plant chestnut, was refined from TCM using advanced extraction techniques(Wang and Yang 2021). Studies have found that SA has a wide range of anti-inflammatory activities. Large number of studies have been reported about SA in the treatment of neurological injuries, tumor cell suppression, cervical spondylosis, spinal cord diseases and other diseases. Zhang *et al.*(Zhang, Fei, Wang et al. 2020) provided evidence that SA treatment is able to play a key role in neuroprotection after traumatic brain injury via the Nrf2 are pathway. Li *et al.*(Li, Xu, Li et al. 2020) displayed that SA inhibited retinoblastoma cell proliferation and induced apoptosis caspase related apoptosis pathway by arresting the cell cycle at the G2/M phase, indicating that SA may have potential as a chemotherapeutic agent. In addition, a study revealed that SA-treated diabetic rat wounds exhibited better granulation tissue, mainly marked fibroblast proliferation, and the wounds were covered by a thick regenerative epithelial layer, indicating that SA can effectively control and improve wound healing in diabetic rats through its anti-inflammatory and antioxidant activities(Zhang, Cao, Sha et al. 2015). It is interesting to note that the results of a recent study suggest that SA exerts promising analgesic effects in rats with bone cancer pain by inhibiting inflammation and microglial activation(Liu, Qin, Wu et al. 2020). Nevertheless, the role of SA in NP has not been explored.

This study aimed to investigate whether SA has a protective effect on NP and its underlying mechanisms. A chronic contraction injury (CCI) mouse model was first established as an animal model of NP. We then examined the changes of animal behavior, microglial polarization, and inflammatory factor secretion after sciatic nerve ligation by administering SA to mice continuously intrathecally.

2 Materials And Methods

2.1 Animal experiments and grouping

Sixty C57BL/6 mice were purchased from Guangdong Animal Medicine Center and were adaptively fed for 1 week. Experiment 1: Thirty mice were randomly divided into three groups (n = 10 per group), including sham + vehicle, CCI + vehicle, CCI + SA (Aimin Pharmaceutical Co., Ltd., Wuhan, China). Mice in the sham group were treated with sham operation, while mice in the CCI and SA treatment groups were treated with CCI surgery. 40 μg/L of SA or vehicle was administrated by intrathecal injection once a day for 5 consecutive days starting from the 7th day after surgery. Experiment 2: Thirty mice were randomly divided into three groups (n = 10 per group): sham + vehicle (0.9% saline), CCI + vehicle, and CCI + SA. 40 μg/L of SA or vehicle was administered daily for 5 consecutive days starting on postoperative day 1. CCI of the sciatic nerve was adopted as an animal model for experiments as described by Bennett *et al*. (Bennett and Xie 1988). Mice were intraperitoneally injected with 10% chloral hydrate (500 mg/Kg), the limbs were fixed after the mice were under anesthesia, and the local skin of the right posterior thigh was disinfected with complexed iodine. A small forceps was used to blunt separate the biceps femoris gap after incision of the skin to expose the sciatic nerve trunk. The sciatic nerve was ligated with a 4 − 0 chromic catgut, the strength of which was ligated to cause mild fluttering of the calf muscles. The wounds were irrigated with medical hydroxide, sterile saline, respectively, and then the fascia and skin were sutured layer by layer using silk sutures. Mice in the sham group were then exposed without ligation of the sciatic nerve for only a few minutes, and other treatments were the same as those in the CCI surgery group. To exclude experimental errors due to human proficiency factors, all modeling procedures were operated by the same person. If hind limb paralysis or death was present, a new mouse was removed and added. Every procedure was approved by the Animal Care and Use Committee of Yue Bei People's Hospital.

2.2 Measurement of paw withdrawal thresholds to mechanical stimuli

The paw withdrawal threshold (PWT) to mechanical stimulation of the hind limbs on the operated side of mice was measured on 1d preoperatively and 6d, 7d, 8d, 9d, 10d, and 11d postoperatively as described by Hargreaves *et al*.(Pyrzanowska, Piechal, Blecharz-Klin et al. 2012). Baselines were measured 1d before surgery, and mice with values outside the 95% CIs of the overall values were removed. The specific steps were as follows: mice were placed on a 30 cm high wire mesh pad (grid 0.25 cm×0.25 cm) for free movement acclimation for 30 min. Stimulating the mouse 3 and 4 inter toe skin from small to large with different von Frey filaments (capable of force generation: 0.31, 0.38, 0.46, 0.6, 1.0, 1.4, 2.0, 4.0 g, respectively) allowed it to curve 90 degrees, and nonresponse was considered if the mouse was not lifting its foot. The previous process was repeated using von Frey filaments with adjacent pressures, and the interval between two measurements should exceed 15 s until its pressure value (g) was recorded when more than five of ten tests had a lifting foot response measured, with the highest threshold set at 4.0 g. The maximum and minimum values were discarded, and 3 measurements were kept for averaging.

2.3 Spinal cord sample collection

Mice were sacrificed by cervical dislocation immediately after deep anesthesia with 2.5% isoflurane using the pain behavioral assay completed 11 days postoperatively, respectively. The skin subcutaneous tissue was incised along the spinous process and a rongeur individually bitten off the bone to expose the spinal cord. Then, the L4-6 spinal cords were carefully removed, and quickly loaded into cryovials and snap frozen in a liquid nitrogen tank before being transferred to a − 80 ℃ freezer for subsequent ELISA and Western blotting and other molecular biological tests.

2.4 Western blotting

Mouse spinal cords were 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 protein concentration values were then accurately measured according to the BCA protein assay kit (Thermo Fisher, USA). Equal proteins were subjected to 10% SDS-PAGE at 70 V for 30 minutes, followed by 90 minutes at 120 V. Proteins migrated to PVDF membranes at 300 Ma for 2 h, and membranes were subsequently blocked in 5% low-fat milk for 2 h. Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated dairy goat anti-rabbit IgG for 1 hour at room temperature. β-actin served as an endogenous contrast. The information of antibodies was listed in Supplementary Table S1. Protein data were visualized according to the application of the ECL Plus Electrochemiluminescence Detection Kit (Pierce, Rockford, IL, USA) and photographed according to the Electrochemiluminescence 3D Imaging System software. Image J mobile phone software was used to quantify and analyze the relative density of spurious bands.

2.5 Enzyme-linked immunosorbent assay (ELISA)

Spinal cord tissues from mice in each group were collected. The levels of tumor necrosis factor-α (TNF-α, ab208348, Abcam, UK), interleukin-1β (IL-1β, RK0006, Abclonal, Wuhan China), and interleukin-6 (IL-6, EM0121, Fine Test, Wuhan, China) were measured by enzyme-linked immunosorbent assay, and the experimental procedures were strictly performed according to the kit instructions.

2.6 Immunohistochemical staining

Spinal cord tissue sections from mice in each group were deparaffinized, hydrated, antigen retrieval, serum blocking, incubation of Iba-1 primary antibodies (ab178846, Abcam, UK) and corresponding secondary antibodies, washing, DAB color development, hematoxylin counterstaining, and mounting of the sections were performed sequentially. The number of cells positive for Iba-1 expression was counted in three randomly selected high-power fields per section under the microscope.

2.7 M1 and M2 typing of microglia by flow cytometry

Cells from spinal cords of mice in each group were collected and counted so that the number of cells per flow tube reached 1×10^6. CD206 (5 μL) and CD86 (2 μL) fluorescein-labeled antibodies (Abcam, UK) were added and incubated for 20 min in the dark after mixing. The cells were centrifuged and then

analyzed by a flow cytometer. Among them, CD86 + labeled M1 microglia and CD206 + labeled M2 microglia.

2.8 Statistical Analysis

SPSS 22.0 and GraphPad prism 8.0 were used for data analysis and mapping. All the data were described as the mean ± SEM. Differences among more than two groups in the above assays were analyzed using one-way ANOVA, with $P < 0.05$ considered significant.

3 Results

3.1 Analgesic effect of SA on sciatic nerve ligation-induced chronic neuropathic pain in mice

Firstly, to determine whether repeated injections of SA could reverse CCI-induced chronic neuralgia, 30 mice were randomly divided into three groups (n = 10 per group): sham + vehicle; CCI + vehicle; CCI + SA. SA (40 μg/L, intrathecal injection) was administered once daily for 5 consecutive days starting on day 7 after surgery. The mechanical withdrawal thresholds (paw withdraw threshold, PWT) of the contralateral and ipsilateral paws of mice in each group were subsequently detected daily. The results showed that there was no significant difference in PWT measured on the day before surgery on the (ipsilateral) hind foot of the nerve-ligated side in the three groups of mice. Compared with sham operation, CCI operation significantly reduced the mechanical pain threshold in CCI mice from the first postoperative day ($P < 0.001$), and it lasted until the end of the 11d observation (Fig. 1B). Whereas the mouse contralateral paw consistently maintained close proximity to the sham group after CCI-induced modeling (P 0.05, Fig. 1C). The mechanical pain threshold of mice that received SA continued to be administered after CCI surgery was significantly improved compared with that of mice in the CCI group, and the effect lasted until the end of the 11d observation ($P < 0.001$, Fig. 1B). However, the mechanical pain threshold of the contralateral paw of the mice did not change during 1–11 d (P 0.05, Fig. 1C). Subsequently, to further explore whether early treatment with SA could inhibit the development of chronic neuralgia induced by CCI, SA was administered intrathecally (40 μg/L) daily for 5 consecutive days starting on postoperative day 1. The results show that the paw withdrawal threshold was conspicuously increased in the ipsilateral paw at 3–8 days of CCI modeling (SA intrathecal injection was stopped on day 5) ($P < 0.0001$, Fig. 1D), without affecting the contralateral paw (P 0.05, Fig. 1E). However, the PWT in the ipsilateral paw of mice was decreased to the CCI model group from the 9th day onwards until the end of the 11th day observation (P 0.05, Fig. 1D). The above studies suggested that repeated SA treatment could prominently increase the reduction of PWT induced by CCI in the ipsilateral paw of mice.

3.2 SA suppressed the activation of the JNK/p-38 signaling pathway in mice with neuropathic pain.

Previous studies have established that the c-jun-N-terminal kinase (JNK)/p38 pathway can attenuate neuroinflammation and NP induced by CCI. Next, the expression of JNK/p38 pathway proteins in the

spinal cords of mice in each group was assessed using Western blotting to analyze whether the JNK/p38 pathway was involved in the alleviation of NP by SA treatment. The results of Fig. 2 suggested that the protein levels of phosphorylation (p)-JNK and p-p38 were preeminently increased in the spinal cords of mice after CCI-induced modeling ($P < 0.001$), whereas the total protein levels of JNK and p38 did not change (P 0.05). Moreover, the upregulation of p-JNK and p-p38 protein levels was materially reversed in the spinal cords of mice after intrathecal administration of SA ($P < 0.001$), whereas the total JNK and p38 protein levels remained unchanged throughout (P 0.05).

3.3 SA promoted the microglia activation in mice with neuropathic pain.

Iba-1 has been confirmed to be a key kinase in the nociceptive pathway and correlated with the activation of microglia. Therefore, we employed immunohistochemistry to evaluate whether intrathecal injection of SA modulates the expression level of Iba-1 in the spinal cord of mice. As we expected, the number of Iba-1-positive cells in the spinal cord of mice after CCI modeling was signally increased, which was reversed by SA treatment (Fig. 3A, $P < 0.001$). Subsequently, the effect of SA on microglial polarization in the spinal cord of CCI mice was further evaluated. We employed flow cytometry to detect the number of cells positively expressing polarization markers of microglia, including CD86 and CD206. The results showed a significant increase in the number of M1 microglia and a decrease in the number of M2 microglia after CCI modeling. Furthermore, intrathecal administration of SA was able to reverse the CCI-induced upregulation of the M1/M2 ratio of microglia (Fig. 3B, $P < 0.001$). Taken together, SA treatment was able to alleviate microglial activation as well as polarization toward M1 type in CCI mice.

3.4 SA attenuates neuroinflammation in the spinal cord induced by neuropathic pain.

Immune related pro-inflammatory factors play an integral role in the formation of NP, such as IL-1β, IL-6, and TNF-α. Western blotting results indicated that TCM component SA preeminently attenuated the protein expression of important inflammatory factors IL-1β, IL-6, and TNF-α in the mouse spinal cord resulting from the CCI model (Fig. 4A, $P < 0.001$). Similarly, ELISA results revealed that the contents of pro-inflammatory factors were strikingly upregulated in the spinal cords of CCI model mice, which were remarkably reversed after intrathecal administration of SA (Fig. 4B, $P < 0.01$). In this part, our results suggest that SA treatment alleviates spinal neuroinflammation induced by NP in CCI mice.

4 Discussion

NP is a pain syndrome resulting from damage to the peripheral or central nervous system and characterized by spontaneous pain, allodynia, and hyperalgesia(Bouhassira 2019). In addition to the pain caused by itself, NP usually causes physiological and psychological abnormalities such as depression, anxiety, and sleep disturbance, which seriously affect patients' quality of life and health(Cavalli, Mammana, Nicoletti et al. 2019). However, current drugs for NP treatment include anxiolytics, antidepressants, adrenal receptor agonists, opioids, local anesthetics as well as NMDA receptor

antagonists, the efficacy of which remains unsatisfactory, and significant side effects limit their use(Szok, Tajti, Nyári et al. 2019). Therefore, it is urgent to find a safe and effective therapeutic drug with few side effects.

Evidence-based clinical studies have shown that although NP is classified as an intractable disease by the World Health Organization (WHO), TCM exhibits several characteristics and advantages in improving the clinical symptoms and improving the quality of life of patients with NP. For example, Yang *et al.*(Yang, Li, Zou et al. 2021) demonstrated that gallic acid, a TCM extracted from natural plants with anti-inflammatory, analgesic effects, attenuates NP in CCI rats via inhibition of P2X7 receptors and subsequent activation of the TNF-α/STAT3 signaling pathway. In addition, there are other TCM also reported to alleviate NP, for example Divanillyl sulfone(Shao, Xu, Chen et al. 2021), Wu-Tou decoction(Zhu, Xu, Mao et al. 2018), resveratrol(Wang, Shi, Huang et al. 2020), astragalin(Wang, Cai, Wang et al. 2020), etc. SA, a triterpenoid saponin sodium salt, is extracted from the dried fruits of the TCM Solanum, and its main efficacy, including anti inflammation, antioxidation, and restoration of capillary permeability, has been available for half a century since the 1960s. Clinically, SA is mainly used for the treatment of functional abnormalities caused by brain trauma, cerebral ischemia, or cerebral edema. Wang *et al.*(Wang, Yang, Ju et al. 2016) found that the combined use of AS and mannitol produced satisfactory results in the treatment of early swelling after upper extremity trauma surgery. Fan *et al.*(Fan, Guo, Xiao et al. 2005) study suggested that AS increased the expression of anti-apoptotic protein Bcl-2 and decreased the expression of protein of Caspase-3, and then protected against reperfusion injury on ischemic brain. Further, SA has also been discovered to exert potent antitumor effects, including breast cancer(Qi, Lv, Meng et al. 2015), liver cancer(Hou, Li, Cui et al. 2019), gastric cancer([Sodium Aescinate Induced Apoptosis of BGC-823 and AGS Cells by Inhibiting JAK-1/STAT-1 Signaling Pathway] 2016). Additionally, a recent study drew our attention. Yang *et al.*(Liu, Qin, Wu et al. 2020) demonstrated that SA alleviated bone cancer pain-induced pain-related behaviors through the increase of proinflammatory cytokine production as well as microglial activation. This experiment serves as an animal model of NP by applying the CCI model, which has similarities with the characteristics of clinical NP and is widely used by the pain science community. The present experimental study demonstrated that the TCM ingredient SA observably attenuated NP in mice resulting from the CCI model in response to decreased PWT in the ipsilateral paw.

The targets of existing drugs to treat NP are mainly neurons. Studies in multiple models of NP have found that NP is a manifestation of abnormal discharges of dorsal horn neurons caused by peripheral sensation(Lançon, Qu, Navratilova et al. 2021). The common NP therapeutics mainly focus on targeting neuronal functions, but the CNS-related side effects nausea, vomiting, dizziness, etc. caused by these drugs greatly limit the use of these analgesic drugs. Therefore, investigating other mechanisms of the occurrence and development of pain and finding safe and effective means of analgesia become urgent clinical needs. Over the past few decades, there has been increasing research on non-neuronal cells, especially concerning glial cells. Spinal glial cells, mainly microglia, play a crucial role in the development and progression of NP(Tozaki-Saitoh and Tsuda 2019). Microglia are widely distributed in the central nervous system under normal physiological conditions. When the central nerve is injured or ischemia,

inflammation occurs, microglia are rapidly activated to change from a resting state to an activated state and constantly activated to proliferate to participate in early pain and inflammation(Tsuda, Koga, Chen et al. 2017). Distinct activation of microglia is evident in multiple models of nervous system pain. Microglial morphology was changed evidently within 24 h of peripheral nerve injury, and microglial activation was markedly increased after 2–3 days of injury(Yi, Liu, Liu et al. 2021). At the same time, activated microglia can release many inflammatory factors, cytokines, proinflammatory factors to produce neuroinflammation ultimately leading to the occurrence of pain(Tsuda 2018). Ye *et al.*(Ye, Lin, Zhang et al. 2021) suggested that quercetin increased mechanical withdrawal threshold in CCI rats by preventing the activation of microglia and astrocytes. Cheng *et al.*(Cheng, Chen, Hsu et al. 2021) found that the expression of GFAP (astrocyte marker) and Iba-1 (microglial marker) in the ipsilateral spinal dorsal horn of CCI mice was conspicuously decreased after intraperitoneal administration of loganin. Immunohistochemistry in this study revealed that Iba-1, a marker of microglia, was indeed significantly elevated in the spinal cord of mice after CCI surgery, and that the elevation of Iba-1 was particularly suppressed by multiple intrathecal administrations of SA. Moreover, intrathecal administration of SA also could reverse CCI-induced microglial polarization toward M1 type. Microglia, being able to be activated by different external stimuli, present two phenotypes in which their functional status is quite different from that of surface markers. Among them, the classical activation pathway generates an M1 phenotype, expresses surface markers such as iNOS and CD86, and exerts pro-inflammatory versus neurotoxic effects(El-Deeb, El-Tanbouly, Khattab et al. 2022). However, the selective activation pathway generates an M2 phenotype, expresses surface markers such as Arg-1 and CD206, and exerts anti-inflammatory and neuroprotective effects(Zhou, Ji,Chen 2020). Studies have demonstrated that microglia are significantly activated and polarized toward M1 type in animal models of CCI induction(Wang, Jiang, Li et al. 2021). Yuan *et al.*(Yuan and Fei 2022) found that lidocaine inhibited M1 microglial polarization (manifested by decreased Iba-1/CD86 positive cells) but promoted M2 microglial polarization (manifested by increased Iba-1/CD206 positive cells) in a CCI-induced NP rat model, which in turn improved NP.

TNF-α, a member of the type transmembrane protein superfamily, is also one of the pro-inflammatory factors that is first upregulated after nerve injury and secondarily upregulated together with other factors(Liu, Zhou, Wang et al. 2017). TNF-α expression was upregulated in peripheral blood, central neurons, microglia, and astrocytes during NP formation(Gao, Bai, Zhou et al. 2020). A previous study found that CCI surgery induced elevated TNF-α production in the lumbar enlargement spinal cord, which peaked on the third day(Zhang, Wang, Zhang et al. 2021). IL-1β and IL-6 are also pro-inflammatory factors that play an important role in NP(Li, Xu,Yang 2017). Intrathecal administration of IL-1β leads to upregulation of inducible NO synthase expression and causes heat pain hypersensitivity in rats(Kanno, Shimizu, Shinoda et al. 2020). In addition, IL-6 can also upregulate substance P and nerve growth factor to sensitize spinal posterior horn neurons to induce hyperalgesia(Li, Zhou, Yang et al. 2022). Studies have shown that following neuroinflammation or nerve injury, microglia are activated and release neural substances and proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, and increase the sensitivity of postsynaptic spinal dorsal horn neurons, ultimately causing hyperalgesia(Yang, Li, Zhang et al. 2020). Our results are consistent with previous studies showing that TNF-α, IL-1β, and IL-6 expressions were

prominently upregulated in the spinal cords of mice subjected to CCI surgery, whereas SA intrathecal injection strikingly reduced the elevated pro-inflammatory factors induced by CCI surgery, indicating that SA treatment can ameliorate the secondary damage from neuroinflammation induced by CCI surgery.

MAPKs are an important class of signaling molecules within the cell that, upon activation by different factors, form their respective transduction pathways, which in turn activate different transcription factors to exert different biological effects(Kassouf and Sumara 2020). As mentioned earlier, there are three classical MAPK pathways: JNK, ERK1/2, p38(Kim and Choi 2010). Among them, p38 and JNK signaling pathways are important signals involved in the regulation of inflammatory responses(Li, Zhang, Ge et al. 2019). Studies have found that JNK and p38 phosphorylated protein expression was conspicuously enhanced in the spinal cord of C57BL6 mice with CCI-induced NP, which is accompanied by microglial activation(Riego, Redondo, Leánez et al. 2018). Besides, JNK and p38 pathway activation dependently encouraged TNF-α-induced inflammatory responses(Liu, Guo, Song et al. 2020). In this experimental study, we found that the phosphorylation levels of JNK and p38 in the spinal cord of mice after CCI were conspicuously upregulated compared with those in the sham group, but the total protein level was unchanged, that is, their transcriptional activity was not affected, which was consistent with previous studies. In addition, JNK and p38 phosphorylated levels were obviously downregulated after SA intrathecal injection.

5 Conclusions

The above data illustrated that SA might suppress the activation of microglia and neuroinflammation by selectively inhibiting the JNK/p38 signaling pathway, which in turn alleviated CCI-induced NP in mice. This study is the first to investigate the role of SA in CCI -induced NP as well as microglia, expand previous knowledge of TCM, and suggest new ideas and targets for the clinical treatment of NP. However, the present study also had some limitations. First, the experiments used a single dose of SA and lacked screening for the optimal dose. We also need to further explore its drug sensitivity to SA by using the JNK/p38 pathway inhibitors. Additionally, to further explore whether the alleviating effect of SA on NP is related to the inhibitory effect on neuronal apoptosis, detection of apoptosis in the dorsal root ganglia of CCI model mice after SA intrathecal injection would be the next issue to be addressed in this study.

Declarations

Ethics approval: Every procedure was approved by the Animal Care and Use Committee of Yue Bei People's Hospital.

Consent for publication: Not applicable.

Availability of data and materials: The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests: The authors declare that they have no conflict of interest.

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Authors' contributions: Wenqiang Xie and Changke Li conceived the project and designed the experiments. Wenqiang Xie, Changke Li, Jie Hou and Qiang Zhang performed the experiments, analyzed the data. XXX wrote and revised the manuscript.

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Figures

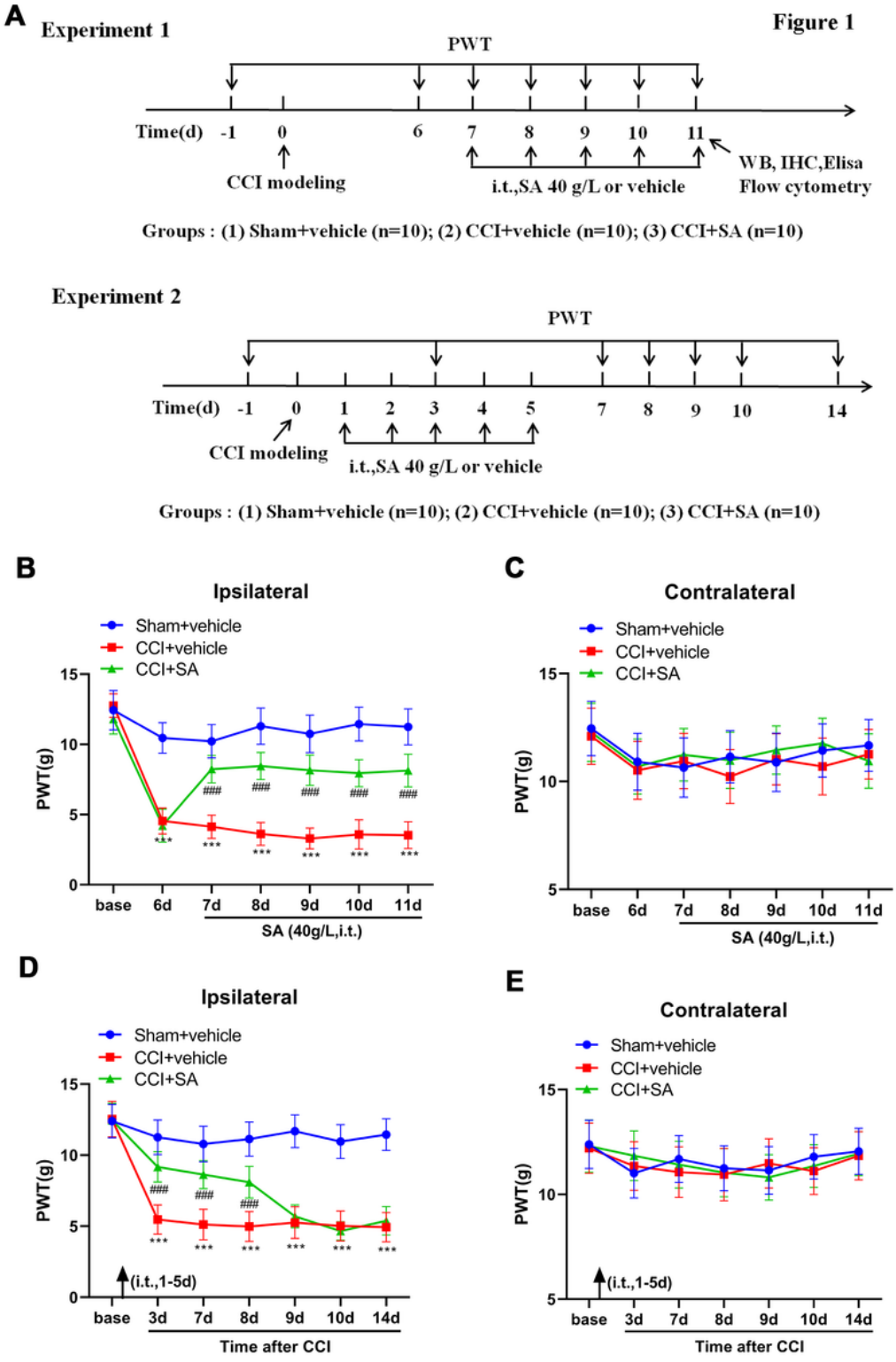

Figure 1

Analgesic effect of SA on sciatic nerve ligation-induced chronic neuropathic pain in mice

A: Experimental design flowchart. Experiment 1: Thirty mice were randomly divided into three groups (n = 10 per group): sham + vehicle; CCI+vehicle; CCI+SA. SA (40 μg/L; intrathecal injection) was administered once daily for 5 consecutive days starting on day 7 after surgery. Experiment 2: Thirty mice were

randomly divided into three groups (n = 10 per group): sham + vehicle; CCI+vehicle; CCI+SA. SA (40 μg/L; intrathecal injection) was administered daily for 5 consecutive days starting on postoperative day 1. **B**: Von Frey monofilaments measurement of the mechanical contraction threshold (paw withdrawal threshold, PWT) of the ipsilateral paw in mice with CCI-induction modeling for 1-11 days (7-11 consecutive daily administrations of SA intrathecally). **C**: Von Frey monofilaments measurement of the mechanical contraction threshold (paw withdrawal threshold, PWT) of the contralateral paw in mice with CCI-induction modeling for 1-11 days (7-11 consecutive daily administrations of SA intrathecally). **D**: Von Frey monofilaments measurement of the mechanical contraction threshold (paw withdrawal threshold, PWT) of the ipsilateral paw in mice with CCI-induction modeling for 1-14 days (1-5 consecutive daily administrations of SA intrathecally). **E**: Von Frey monofilaments measurement of the mechanical contraction threshold (paw withdrawal threshold, PWT) of the contralateral paw in mice with CCI-induction modeling for 1-14 days (1-5 consecutive daily administrations of SA intrathecally). Data are shown as mean ± SEM. ***P < 0.001 *vs* sham+vehicle group. ###P < 0.001 *vs* CCI+vehicle group. n=10.

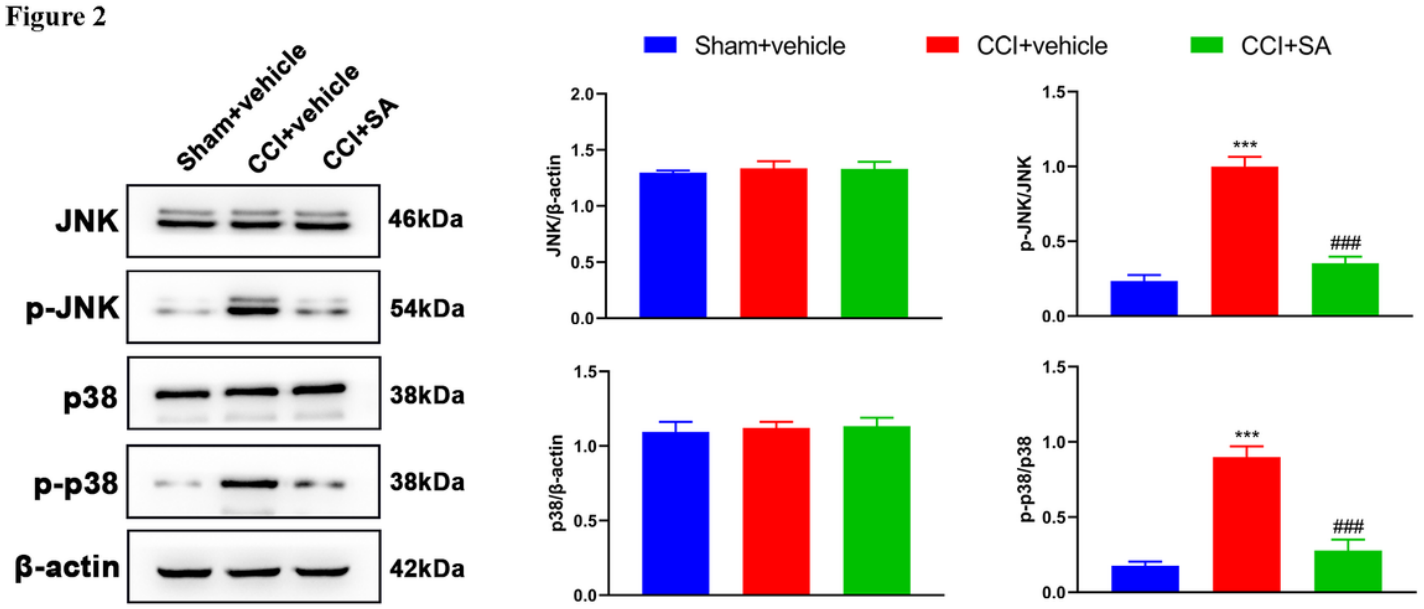

Figure 2

SA suppressed the activation of JNK/p-38 signaling pathway in mice with neuropathic pain.

Western blotting measurement of the protein levels of phosphorylation (p)-JNK, p-p38, JNK and p38 in the spinal cords of mice in each group, including sham + vehicle; CCI+vehicle; CCI+SA. SA (40 μg/L; intrathecal injection) was administered once daily for 5 consecutive days starting on day 7 after surgery. Mice were sacrificed by decapitation under deep anesthesia with chloral hydrate 11 days later, and L4-6 spinal cords were removed for protein detection. Data are shown as mean ± SEM. ***P < 0.001 *vs* sham+vehicle group. ###P < 0.001 *vs* CCI+vehicle group. n=3.

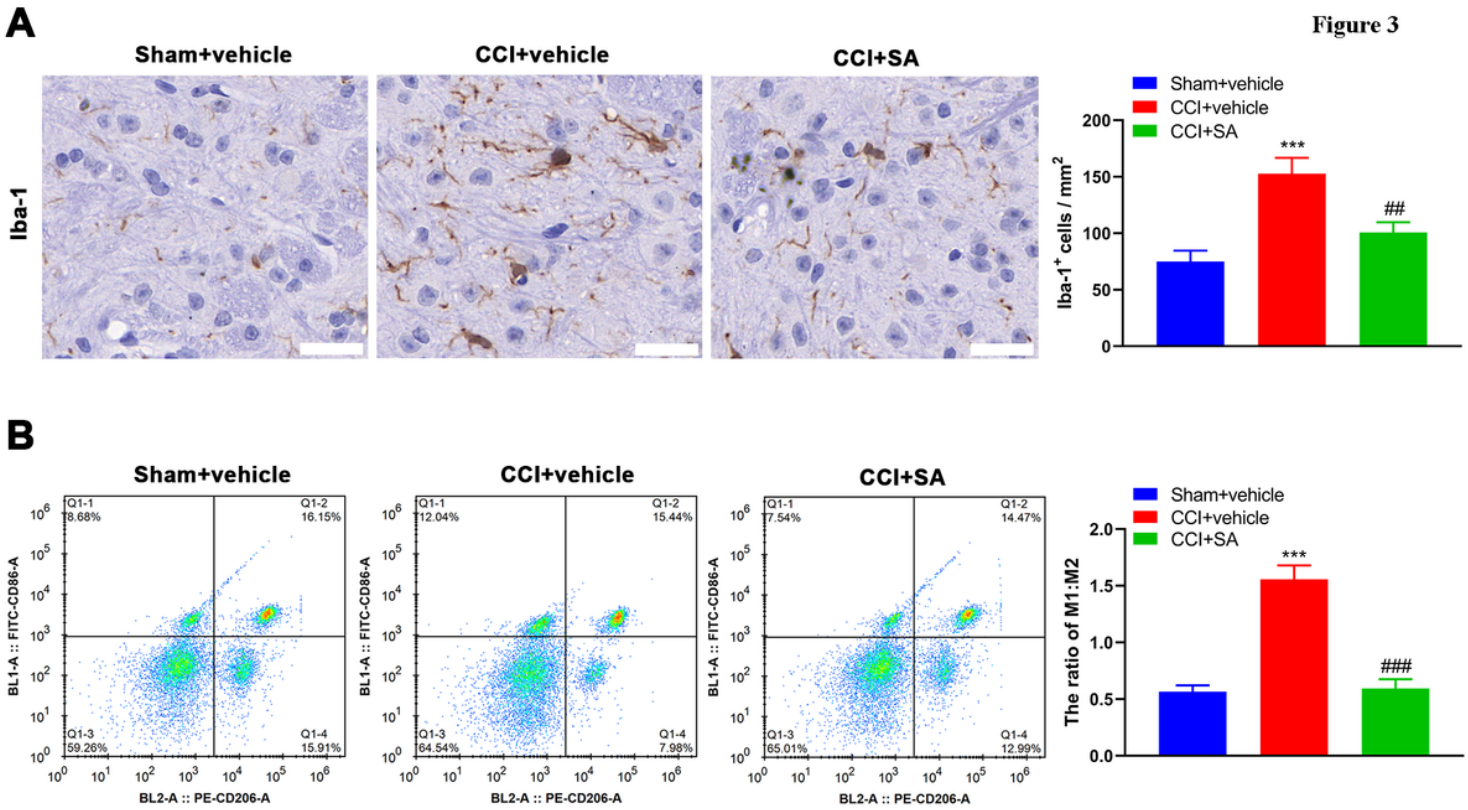

Figure 3

SA promoted microglia activation in mice with neuropathic pain.

A: Immunohistochemistry measurement of the positive expression of Iba-1 in the spinal cord of mice in each group. **B**: Flow cytometry detection of the polarization of microglia in the spinal cord of mice in each group. CD86 was M1 marker, M1 was seen in the upper left quadrant; CD206 was M2 marker, M2 was seen in the lower right quadrant. Data are shown as mean ± SEM. ***P< 0.001 *vs* sham+vehicle group. ###P < 0.001 *vs* CCI+vehicle group. n=3.

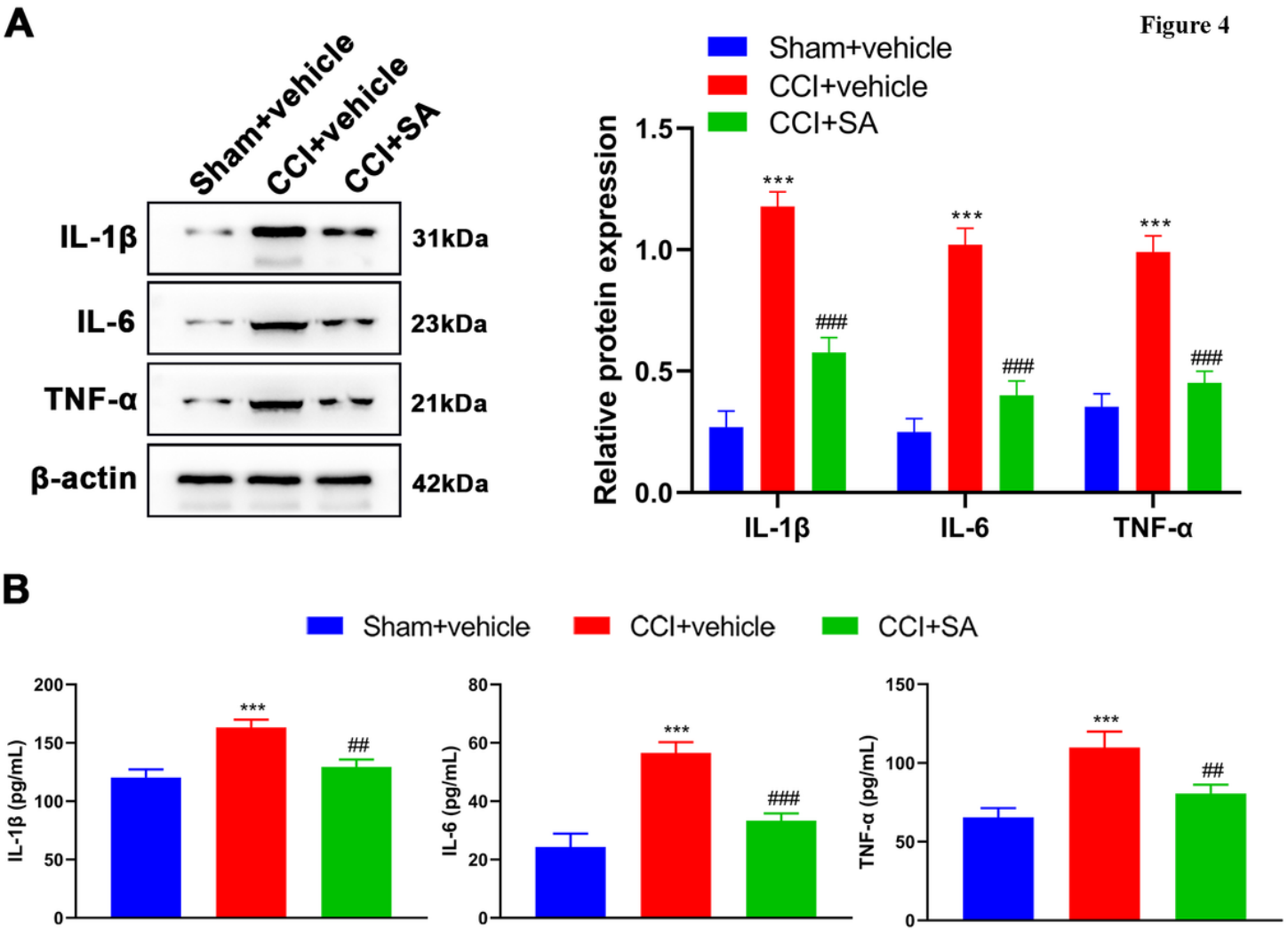

Figure 4

SA attenuates neuroinflammation in the spinal cord induced by neuropathic pain.

A: Western blotting assessment of the protein levels of IL-1β, IL-6 and TNF-α in the spinal cord of mice in each group. **B**: ELISA assessment of the contents of IL-1β, IL-6 and TNF-α in the spinal cord of mice in each group. Data are shown as mean ± SEM. ***$P < 0.001$ *vs* sham+vehicle group. ##$P < 0.01$ and ###$P < 0.001$ *vs* CCI+vehicle group. n=3.

Supplementary Files

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- TableS1.docx