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The analgesic effects of β-elemene in rats with neuropathic pain by inhibition of spinal astrocytic ERK activation

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Abstract

Neuropathic pain takes a heavy toll on individual well-being, while current therapy is far from desirable. Herein, we assessed the analgesic effect of β-elemene, a chief component in the traditional Chinese medicine Curcuma wenyujin, and explored the underlying mechanisms at the level of spinal dorsal horn (SDH) under neuropathic pain. A spared nerve injury (SNI)-induced neuropathic pain model was established in rats. Intraperitoneal injection (i.p.) of β-elemene was administered for 21 consecutive days. Mechanical allodynia was explored by von Frey filaments. The activation of the mitogen-activated protein kinase (MAPK) family (including ERK, p38, and JNK) in spinal neurons, astrocytes, and microglia was evaluated using immunostaining 29 days after SNI surgery. The expression of GFAP, Iba-1, p-ERK, p-JNK, and p-p38 within the SDH was measured using immunoblotting. The levels of proinflammatory cytokines (including TNF-α, IL-1β, and IL-6) were measured with ELISA. The levels of oxidative stress indicators (including MDA, SOD, and GSH-PX) were detected using biochemical tests. Consecutive i.p. administration of β-elemene relieved SNI-induced mechanical allodynia (with an EC50 of 16.40 mg/kg). SNI significantly increased the expression of p-ERK in spinal astrocytes but not microglia on day 29. β-elemene reversed spinal astrocytic ERK activation and subsequent upregulation of proinflammatory cytokines in SNI rats, with no effect on the expression of p38 and JNK in spinal glia. β-elemene also exerted antioxidative effects by increasing the levels of SOD and GSH-PX and decreasing the level of MDA. Our results suggest that SNI induces robust astrocytic ERK activation within the SDH in the late phase of neuropathic pain. β-elemene exerts remarkable analgesic effects on neuropathic pain, possibly by inhibiting spinal astrocytic ERK activation and subsequent neuroinflammatory processes. Our findings suggest that β-elemene might be a promising analgesic for the treatment of chronic pain.

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Introduction

Neuropathic pain is a common type of pathological pain resulting from nerve injury, including diabetic neuropathy, drug-triggered neuropathy and traumatic nerve injury.[1] It severely compromises the quality of life of patients and remains a tough challenge to global public health.[2] Traditional pharmacotherapy for neuropathic pain, including opioids, antiepileptics, antidepressants and nonsteroidal anti-inflammatory drugs, provides favourable analgesic efficacy.[3] However, these drugs are limited due to side effects, including tolerance, addiction and behaviour disorders.[4-6] The dilemma in looking for ideal analgesics pushes Chinese scientists to draw inspiration from traditional Chinese medicine. With the development and modernization of herbal medicine, an increasing number of active ingredients with analgesic properties have been identified from natural products, providing alternative measures for pain management.[7,8]

β-elemene (Figures 1(a) to (c)) is a sesquiterpene compound isolated from *Curcuma wenyujin* that has been used for centuries in traditional Chinese medicine.[9,10] Owing to its proven efficacy and safety, β-elemene has been approved by the Chinese Food and Drug Administration for human cancer treatment, especially for solid tumours and malignant effusions.[11,12] β-Elemene exerts antitumour effects *by* inhibiting tumour proliferation and metastasis, inducing cell apoptosis, and enhancing cancer sensitivity to chemotherapy.[13] Of note, it has also been adopted to alleviate cancer pain in the clinic,[14,15] probably due to its antioxidative and immunosuppressive potency.[16] However, whether β-elemene can attenuate neuropathic pain remains elusive.

Accumulating evidence suggests that neuropathic pain results from neuroinflammation in the spinal dorsal horn (SDH). It is triggered by the stimuli-dependent release of glial activators from primary afferent neurons and is characterized by spinal glial activation and subsequent pronociceptive mediator production. Glial mediators, such as TNF-α, IL-1β and IL-6, modulate excitatory synaptic transmission via glia-neural interactions, thus leading to central sensitization.[17] As canonical intracellular signalling molecules, the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), is activated by nerve injury, linking spinal glial activation and the generation of inflammatory factors.[18] Upon peripheral nerve damage, microglia (within days) first respond, followed by astrocytes (within days to weeks), which play a key role in the induction and maintenance of pathological pain, respectively.[19,20] As an immunoregulation agent, β-elemene has been reported to alleviate inflammatory brain diseases.[21–24] Several lines of evidence also implicate the involvement of MAPK in the antineoplastic, anti-inflammatory and antioxidative activities of β-elemene.[22,25–28] Considering these findings, we propose that β-elemene may inhibit spinal neuroinflammation under neuropathic pain conditions via glial MAPK pathways.

Thus, the aim of the present study was to explore the involvement of spinal inflammatory mechanisms in β-elemene-induced analgesia via a sciatic nerve injury (SNI) rat model. First, the anti-nociceptive effect of β-elemene was assessed. Then, the effects of β-elemene on the activation of spinal glia (including astrocytes and microglia) and the MAPK family (including ERK, JNK, and p38) were investigated. Next, the effects of β-elemene on proinflammatory cytokine generation (including TNF-α, IL-1β, and IL-6) was examined. Finally, the potential antioxidative property of β-elemene in SNI rats was also examined by the measurement of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX).

Materials and methods

Animals and experimental design

Male Sprague–Dawley rats (200–250 g) used in this study were provided by the Experimental Animal Center of The Fourth Military Medical University (Xi'an, China). The rats were housed with a 12 h light-dark cycle with free access to food and water. All experiments were performed according to the ethical guidelines of the International Association for the Study of Pain[29] and approved by The Fourth Military Medical University Committee on Animal Care and Use. All efforts were made to minimize the number of animals used and animal suffering. Animals selected for tissue collection were euthanized with an overdose of 2% pentobarbital sodium at a dose of 100 mg/kg, while those selected for surgery were deeply anaesthetized using 2% pentobarbital sodium (40 mg/kg).

The experimental design was as follows. Seventy-two rats were equally divided into six groups: sham + veh, SNI + veh, SNI + β-elemene (5 mg/kg), SNI + β-elemene (10 mg/kg), SNI + β-elemene (20 mg/kg) and SNI + β-elemene (40 mg/kg). Herein, β-elemene injection (Huali Jingang Pharmaceutical Co., Ltd, Liaoning, China) was intraperitoneally (*i.p.*) according to previous literature.[30,31] At Day 1, the baseline paw withdrawal threshold (PWT) was measured via the *von* Frey filaments (VFF) test. Then, neuropathic pain was

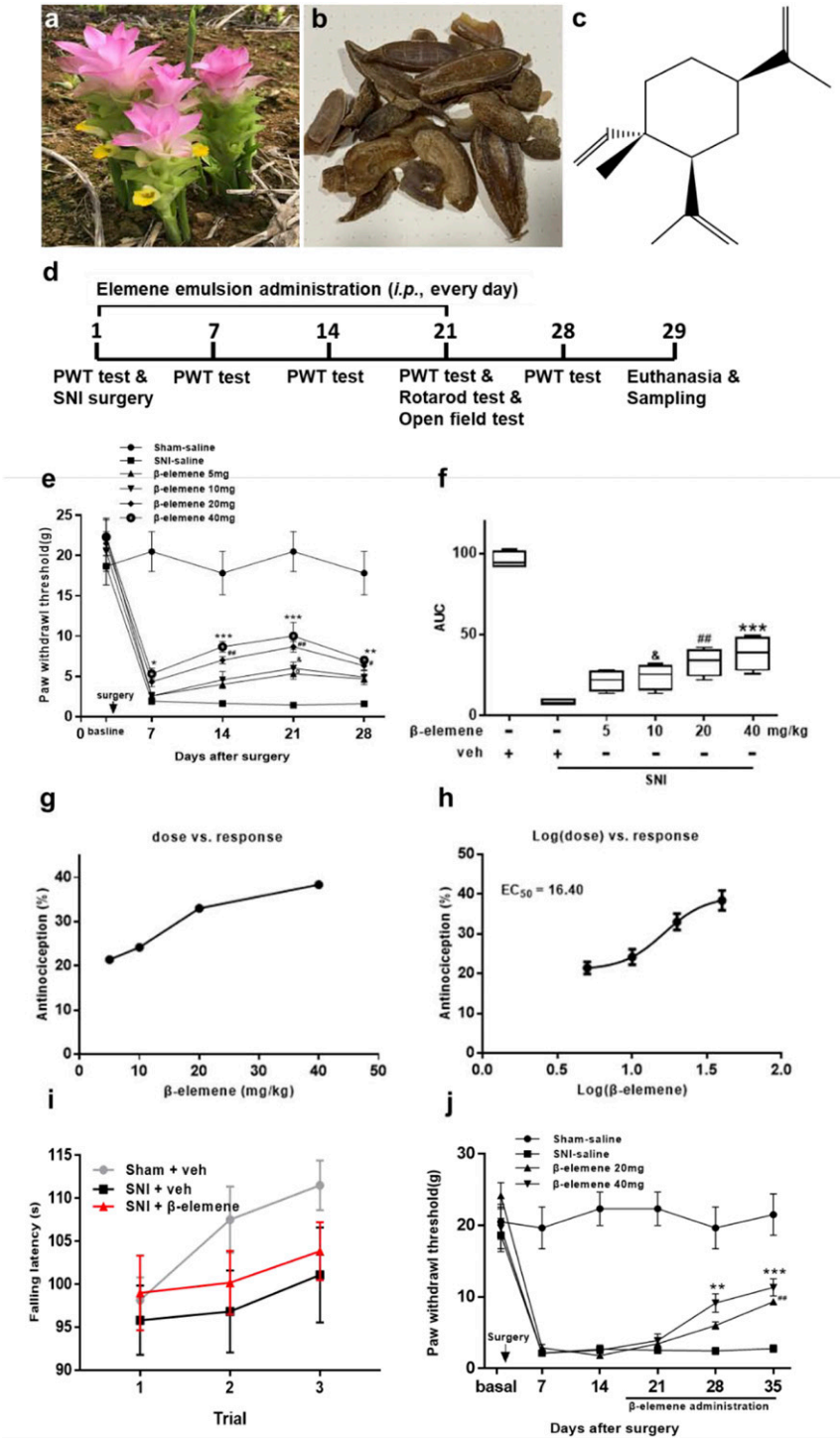

Figure 1. Effects of consecutive *i. p.* β-elemene administration on SNI-induced mechanical allodynia. (a) The botanical source of the Chinese medicine *Curcumae Radix*. (b) Pieces of the Chinese medicine *Curcumae Radix*. (c) Chemical structure of β-elemene. (d) Schematic diagram demonstrating the timeline for the behavioural experiment. (e) Analgesic effect of beta-elemene at different doses. However, β-elemene dose-dependently alleviated mechanical allodynia in SNI rats, repeated ANOVA. (f) The AUCs of these groups were measured for statistical analysis, one-way ANOVA. (g) and (h) show the dose-effect and log (dose)-effect curves for the analgesic effects of β-elemene, respectively. The EC_{50} of β-elemene on SNI-induced mechanical allodynia was 16.40 mg/kg. (i) No significant change in falling latency in the rotarod test among the three groups, repeated ANOVA. (j) Effects of consecutive (i). p. β-Elemene administration on established SNI-induced mechanical allodynia. Begin injection of beta-elemene on day 14 after surgery. n = 6 rats in each group, $^{\&}p < 0.05$, SNI + β-elemene (10 mg/kg) group versus SNI + veh group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, SNI + β-elemene (20 mg/kg) group versus SNI + veh group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, SNI + β-elemene (40 mg/kg) group versus SNI + veh group.

induced using sciatic nerve injury. β-elemene or saline was administered every day from Day 1 to Day 21. After SNI surgery, the PWT was measured on Days 7, 14, 21, and 28. At Day 21, the rotarod test was also performed to evaluate the effect of β-elemene on motor coordination. On Day 29, the rats were euthanized. We observed that on Day 29, both 20 mg/kg and 40 mg/kg of β-elemene could significantly alleviate neuropathic pain, and there was no significant difference in the PWT of rats in these two groups (Figure 1(e)). Considering the potential side effects[32] of this drug, the rats treated with a lower, effective dose (20 mg/kg) was selected for subsequent morphological and biochemical experiments. Three rats in each group were processed for lumbar spinal cord sampling for immunoblotting and enzyme-linked immunosorbent assay (ELISA). The others were perfused with 4% paraformaldehyde (PFA), and the lumbar spinal cord was sampled for immunostaining.

To test the analgesic effects of β-elemene in the later phase of neuropathic pain, 22 rats were equally divided into four groups: sham + veh, SNI + veh, SNI + β-elemene (20 mg/kg) and SNI + β-elemene (40 mg/kg). β-Elemene was administrated every day from Day 14 to Day 28 after SNI surgery, and the PWT of rats in each group was measured.

SNI surgery

The rats were deeply anaesthetized. As previously described,[33] an incision was made in the hind leg to expose the biceps femoris muscle. Then, the sciatic nerve and its three terminal branches (the tibial, common peroneal, and sural nerves) were exposed. The tibial and common peroneal nerves were tightly ligated with 6.0 silk suture and sectioned distal to the ligation. The muscle and skin were closed in two separate layers. For sham surgery, the sciatic nerve was only exposed.

Behavioural tests

The mechanical threshold was examined using the VFF test. Rats were habituated in the testing apparatus until calming down. As previously described,[34] a series of VFFs with increasing forces from 1.4 g to 26 g were applied to the lateral plantar surface of the hindpaw 5 times, each for 5–8 s with a 5 min interval. The minimal force causing withdrawal responses at least 3 times in five stimulations was considered the PWT. Positive signs for withdrawal behaviours included ipsilateral rear leg vibrating, withdrawal, nibbling and vocalization.

The area under the time-course curve (AUC) values was used to calculate the summed effects of different treatments from Day 1 to Day 28. To calculate the antinociception percentage, the mean values of the AUCs of rats in the sham + veh group (AUC_{sham}) and SNI + veh group (AUC_{BCP}) were first measured. Then, the antinociception (%) of individual animals in the β-elemene-treated groups (AUCi) was calculated with the following formula: antinociception (%) = $(AUC_i - AUC_{SNI})/(AUC_{sham}-AUC_{SNI}) \times 100$. Next, the intragroup mean values of antinociception (%) in the SNI + β-elemene groups were calculated. The β-elemene dosages were transformed into logarithmic doses with GraphPad Prism software (CA, USA), and the nonline fit was performed to build the log (dose)-effect curve.[16] Based on the log (dose)-effect curve, the median effective concentration (EC50) of the analgesic effect of β-elemene was calculated.

Motor coordination was measured using the rotarod test. As previously described,[35] rats were habituated in the behavioural room until calming down. Before the test, the rats were trained for three trials on a rotarod apparatus (Shanghai Biowill Co. Ltd, Shanghai, China) rotating at a constant speed of 5 revolutions per minute (rpm). In the test, rats were placed on the rotating rod starting at 3 rpm and progressing to a maximum of 30 rpm for three trials. The falling latency was measured.

Immunofluorescence staining

On Day 29, the anaesthetized rats were transcardially perfused with 100 mL of 0.01 M phosphate-buffered saline (PBS, pH 7.2) and then 500 mL of 0.1 M phosphate buffer (PB, pH 7.2) containing 4% paraformaldehyde. The lumbar 4-6 segments of the spinal cord were harvested according to the termination of dorsal roots, postfixed in 10% PFA solution for 48 h, embedded in paraffin wax (Shanghai Huayong Olefin Co., Ltd, Shanghai, China), and then serially cut into 5 μm thick sections on a microtome (Leica RM2016, Shanghai, China). All sections were divided into nine series, which were subsequently subjected to double immunofluorescence staining according to a previous study[36] for p-ERK and NeuN, p-ERK and glial fibrillary acidic protein (GFAP), p-ERK and ionized calcium-binding adaptor molecule (Iba-1), p-JNK and NeuN, p-JNK and GFAP, p-JNK and Iba-1, p-p38 and NeuN, p-p38 and GFAP, and p-p38 and Iba-1.

First, paraffin sections were dewaxed, hydrated and placed in a box filled with ethylene diamine tetraacetic acid (EDTA) antigen repair buffer (pH 9.0, Servicebio, Hubei, China) to repair the antigen in a microwave oven. After that, an autofluorescence quenching agent (Servicebio) was added, and the sample was sealed with 5% BSA (Servicebio) for 30 min at room temperature (RT). An anti-incubation (Servicebio) was added and incubated overnight at 4°C.

Then, the sections were incubated with primary antibodies diluted in 0.01 M PBS overnight at 4°C: mouse anti-GFAP (1:3000; GB12096; Servicebio); rabbit anti-Iba-1 (1:2500; GB12105; Servicebio); rabbit anti-NeuN (1:5000; GB11138-1; Servicebio). After three washes with 0.01 M PBS, the sections were incubated with the corresponding secondary antibodies for 1 h at RT: FITC-conjugated goat anti-rabbit IgG (1:500; GB23303; Servicebio) and FITC-conjugated goat anti-mouse IgG (1:500; GB22401; Servicebio). After three washes, the sections were placed in a box filled with

EDTA antigen repair buffer to separate the combined primary and secondary antibodies from the tissue in a microwave oven.

Next, the sections were reincubated with the following primary antibodies overnight at 4°C: rabbit anti-p-ERK (1:500; GB11507; Servicebio); rabbit anti-p-p38 (1:500; GB13006-1; Servicebio); and rabbit anti-p-JNK (1:300; GB13019-1; Servicebio). After washing, these sections were reincubated with Cy3-conjugated goat anti-rabbit IgG (1:500; GB21303; Servicebio) for 1 h at RT. The sections were then rinsed and cover-slipped with anti-fading solution.

The sections were observed under a confocal laser scanning microscope (Eclipse Ti, Tokyo, Japan) with appropriate laser beams and filters for FITC (excitation 488 nm; emission 510–530 nm) and Cy3 (excitation 510–560 nm; emission 590 nm) after each immunostaining. Digital images were captured using Eclipse C2 software (Nikon). Six sections from three rats (2 sections per rat, 20 x) in each group were selected for immunofluorescence staining analysis. In each section, two microscopic images of the SDH (100 x) were randomly taken, and the number of cells of interest was counted.

Western blotting

Spinal cord tissues were harvested in cold PBS. Both the ipsilateral and contralateral parts of the SDH were separated. The tissues were equally divided into two parts for immunoblotting and ELISA. For immunoblotting, total protein was prepared according to our previous study.[34] Subsequently, 30 μg of protein from each sample was quantitatively measured with bicinchoninic acid protein assay (Thermo Scientific; IL, USA), subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, United States). After blocking with 5% DifcoTM skim milk in Tris-buffered saline with Tween (TBST) for 2 h, the membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-GFAP (1:1000; YM3059; Immunoway); rabbit anti-Iba-1 (1:500; Bs-1363R; Bioss); rabbit anti-p-ERK (1:1000; YP0101; Immunoway); rabbit anti-p-p38 (1:1000; YP0203; Immunoway); and rabbit anti-p-JNK (1:1000; YP0843; Immunoway). The immunoblots were then incubated with the following secondary antibodies: HRP-conjugated goat anti-rabbit (1:10,000; S004F; TDY Biotech Co., Beijing, China) or goat anti-mouse (1:10,000; S001F; TDY Biotech Co., Beijing, China). To verify equal loading, we also probed the membranes with rabbit anti-GAPDH (1:5000; TDY052C; TDY Biotech Co., Beijing, China). Bands were visualized using an enhanced chemiluminescence (ECL) detection method (Amersham Pharmacia Biotech, NJ, USA) and exposed to film. The scanned images were quantified and analysed with ImageJ software (National Institutes of Health, MD, USA). The protein levels were normalized against those of GAPDH and expressed as fold changes relative to the sham group.

Measurement of oxidative stress indicators

The levels of MDA, SOD and GSH-PX activities in the SDH were determined with commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The procedures were performed in accordance with the corresponding kit protocols.[37]

ELISA

The levels of TNF-α, IL-1β and IL-6 in the SDH were measured using commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute) according to standard procedures.[38]

Data analyses

Data are presented as the means ± standard errors of the means (SEM) and were analysed by researchers who were blinded to the experimental design. Repeated-measure of analysis of variance (ANOVA) followed by LSD *post hoc* tests was used for multiple comparisons for consecutive behavioural tests (SPSS 17.0). One-way ANOVA followed by LSD *post hoc* tests were used in immunoblotting, immunostaining and ELISA analyses. $p < 0.05$ was considered statistically significant.

Results

Consecutive *i. p.* of β-elemene relieved SNI-induced mechanical allodynia in a dose-dependent manner

To explore the effect of β-elemene on SNI-induced mechanical allodynia, the drug was firstly administrated in the early phase of neuropathic pain (from Day 1 to Day 21 on a daily basis) (Figure 1(d)). As shown in Figure 1(e), SNI-treated rats exhibited a significantly lower PWT than sham rats. Lower doses of β-elemene (5 and 10 mg/kg) exhibited mild, but not significant (except the dose of 10 mg/kg on Day 21) analgesic effects in SNI-treated rats, while higher doses (20 and 40 mg/kg) exhibited remarkable analgesic effects, which persisted until 7 days after drug withdrawal on Day 28. The effect of β-elemene on SNI-induced PWT changes was also counted in accordance with the dose–response and log (dose)-response curves (Figures 1(g) and (h)), through which the EC_{50} of β-elemene was calculated. Since the EC_{50} of β-elemene was 16.40 mg/kg, the dose at 20 mg/kg (close to EC_{50}) was chosen for subsequent experiments on its analgesic mechanisms.

In addition, in order to test the analgesic role of beta-elemene on established neuropathic pain, we started the

injection of β-elemene in the later phase of neuropathic pain (from Day 14 to Day 28 on a daily basis) after SNI surgery, and measured the PWT of animals in each group on Day 21 and 28. Interestingly, β-elemene at the dose of 40 mg/kg showed significant analgesic effects 14 days after initial administration ($p < 0.01$), and β-elemene at the dose of 20 mg/kg exhibited significant analgesic effects on Day 21 after administration (Figure 1(j)). Fewer side effects are always expected for an ideal analgesic. To determine whether β--elemene (20 mg/kg) elicited locomotion dysfunction in SNI rats, rotarod and open field tests were performed to examine animal locomotion coordination. For rotarod test, there was no significant group difference in the falling latency during the three trials among the three groups (Figure 1(i), $P > 0.05$).

p-ERK was primarily expressed in spinal astrocytes of SNI rats on Day 29

Accumulating evidence supports the notion that spinal MAPKs contribute to the development of neuropathic pain. After nerve injury, p38, JNK and ERK are differentially activated in spinal neurons and glial cells, leading to neural plasticity and neuroinflammatory processes.[18] Since the main purpose of this study was to explore the role of spinal MAPKs in β-elemene-induced analgesia, the expression of activated MAPKs (the phosphorylated form) in different types of spinal cells of SNI rats on Day 29 was first clarified. Our immunostaining data showed that 29 days after SNI injury, more p-ERK-positive cells expressed GFAP (82.18 ± 2.61% of total p-ERK-expressing cells) than Iba-1 (28.11 ± 2.52%) within the ipsilateral SDH but not NeuN (Figure 2 and Figure 3); p-JNK-positive cells expressed both NeuN (54.41 ± 2.95% of total p-JNK-expressing cells) and GFAP (32.73 ± 2.81%) but not Iba-1 (Supplemental Figure 1 and Figure 4); and p-p38-positive cells expressed both NeuN (62.84 ± 1.65% of total p-p38-expressing cells) and Iba-1 (56.56 ± 2.42%) but not GFAP (Supplemental Figure 2 and Figure 4). All these data suggest that during the late phase of neuropathic pain, different MAPK subtypes are expressed in different spinal cells.

β-elemene inhibited SNI-induced astrocytic ERK activation

Since p-ERK was mainly expressed in spinal astrocytes in the late phase of neuropathic pain, we detected the effect of β-elemene on astrocytic ERK activation 29 days after SNI. Immunostaining analyses showed that SNI significantly

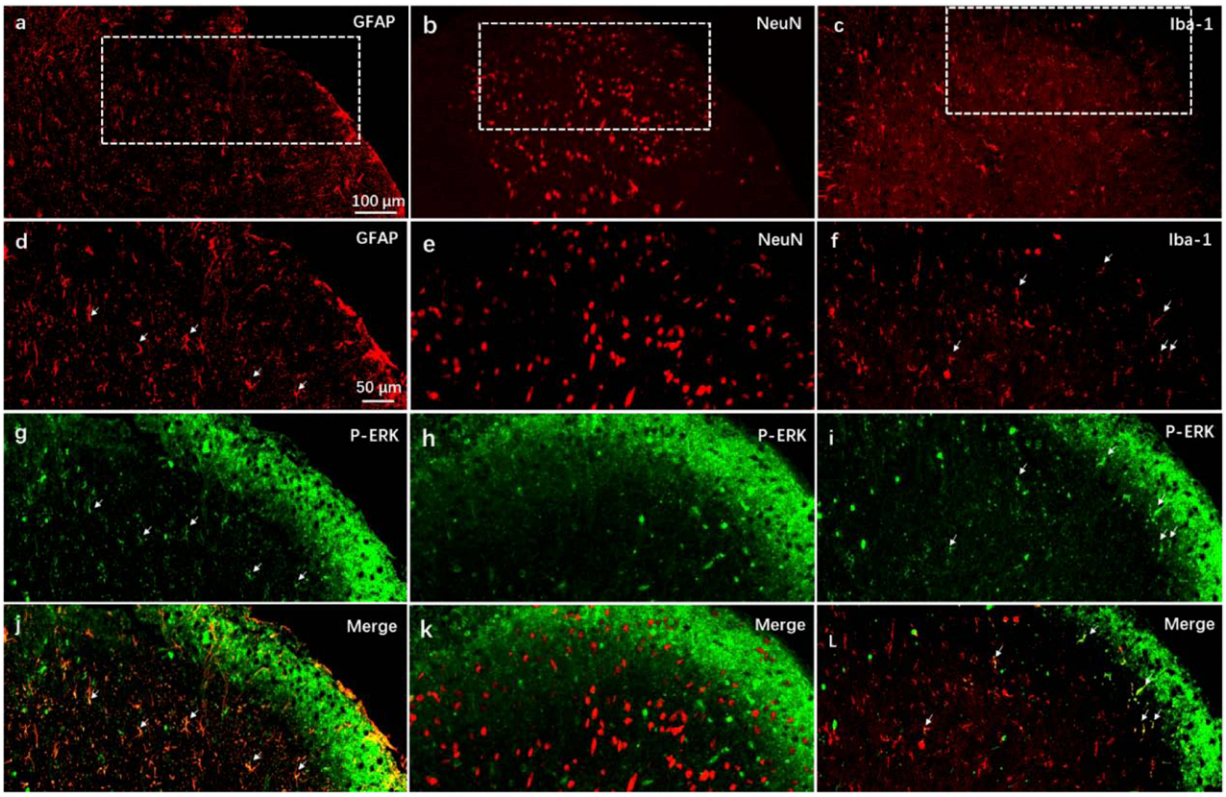

Figure 2. p-ERK was primarily expressed in spinal astrocytes and microglia of SNI rats on Day 29. Microphotographs indicating double-immunofluorescence histochemistry for p-ERK (green) and GFAP (a, d; red) or NeuN (b, e; red) or Iba-1 (c, f; red) immunoreactivities within the ipsilateral spinal dorsal horn of SNI rats on Day 29. The framed areas in images (a, b and c) were magnified in images (d-f, g-i and j-l), respectively. White arrows show double-labelled cells in the images of each set. Bars = 100 μm in images (a, b and c) and 50 μm in images (d-i).

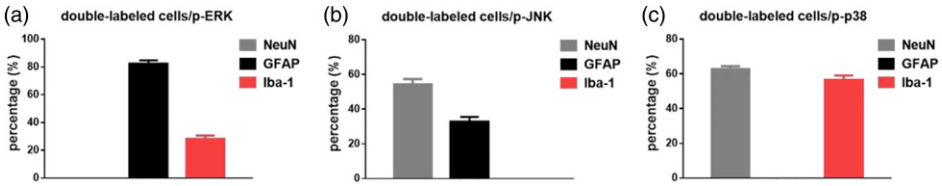

Figure 3. Bar graphs showing the expression of p-ERK (a), p-JNK (b) and p-p38 (c) in neurons, astrocytes and microglia within the ipsilateral spinal dorsal horn in SNI rats on Day 29. n = 6 sections from three rats in each group.

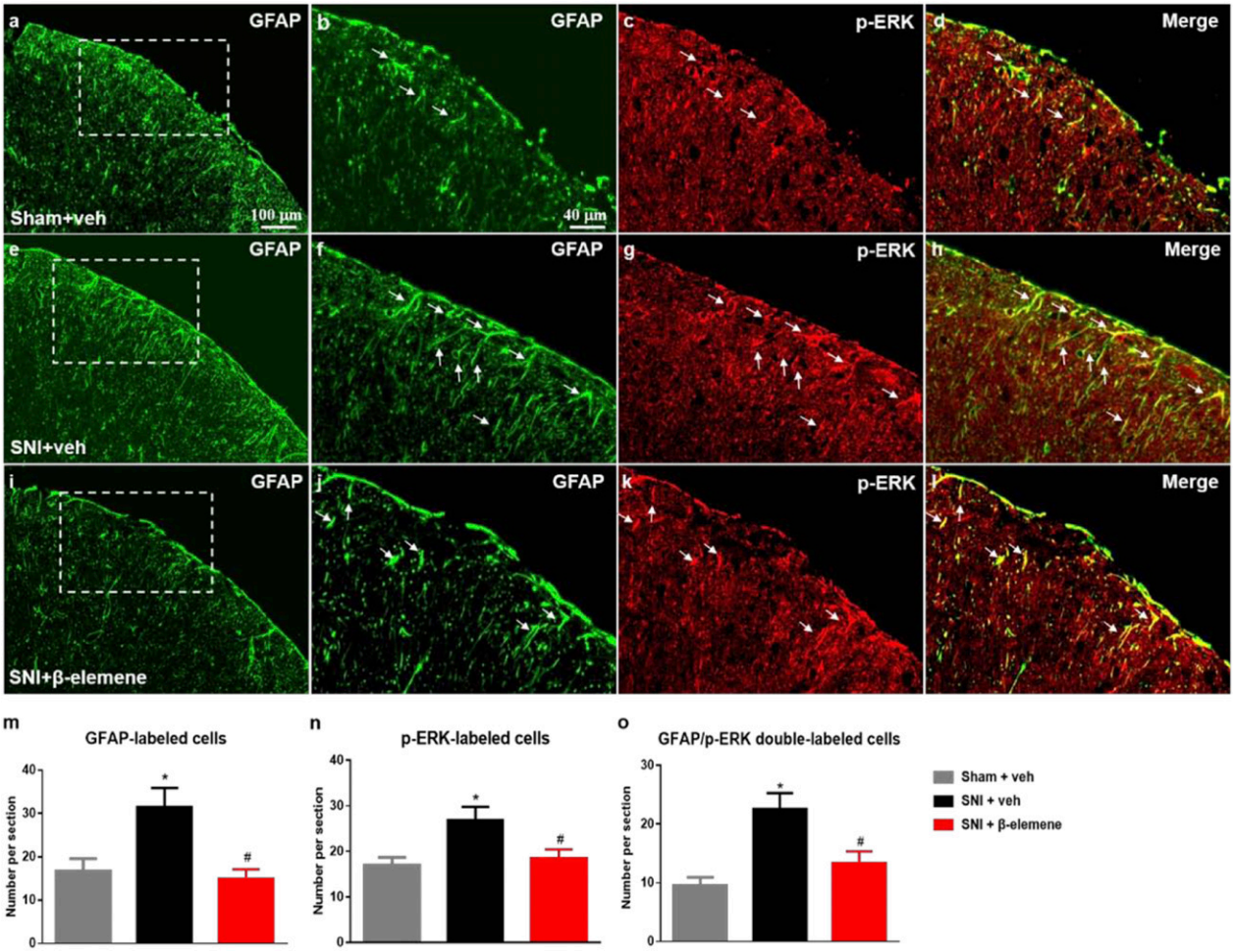

Figure 4. β-elemene inhibited SNI-induced spinal astrocytic ERK activation. SNI induced a marked increase in the number of GFAP, p-ERK and GFAP/p-ERK double-labelled cells (a-d, e-h) within the ipsilateral spinal dorsal horn on Day 29; β-elemene decreased the number of GFAP, p-ERK and GFAP/p-ERK double-labelled cells (i–l) in SNI rats. The framed area in images (a, e and i) was magnified in images (b-d, e-h and i-l), respectively. White arrows show double-labelled cells in the images of each set. Bars = 100 μm in images (a, e and i) and 40 μm in images (b-d, e-h and i-l). The numbers of GFAP, p-ERK and GFAP/p-ERK double-labelled cells among the three groups are shown in m, n and o, respectively (*n* = 6 sections from three rats in each group).

increased the number of GFAP-immunoreactivity (-ir), pERK-ir and GFAP/p-ERK double-labelled cells within the ipsilateral SDH compared to the sham group; β-elemene obviously decreased the number of GFAP-ir and pERK-ir as well as GFAP/p-ERK double-labelled neurons compared to the SNI group (Figure 4). In addition to astrocytes, SNI also increased the number of spinal microglia. However, β-elemene did not influence the number of Iba-1-ir or Iba-1/p-ERK double-labelled cells in SNI rats (Figure 5). All these data indicate that spinal astrocytes are recruited in the late phase of neuropathic pain and that β-elemene inhibits SNI-induced astrocytic ERK activation.

Our immunostaining data showed that p-JNK was expressed in both spinal neurons and astrocytes in SNI rats.

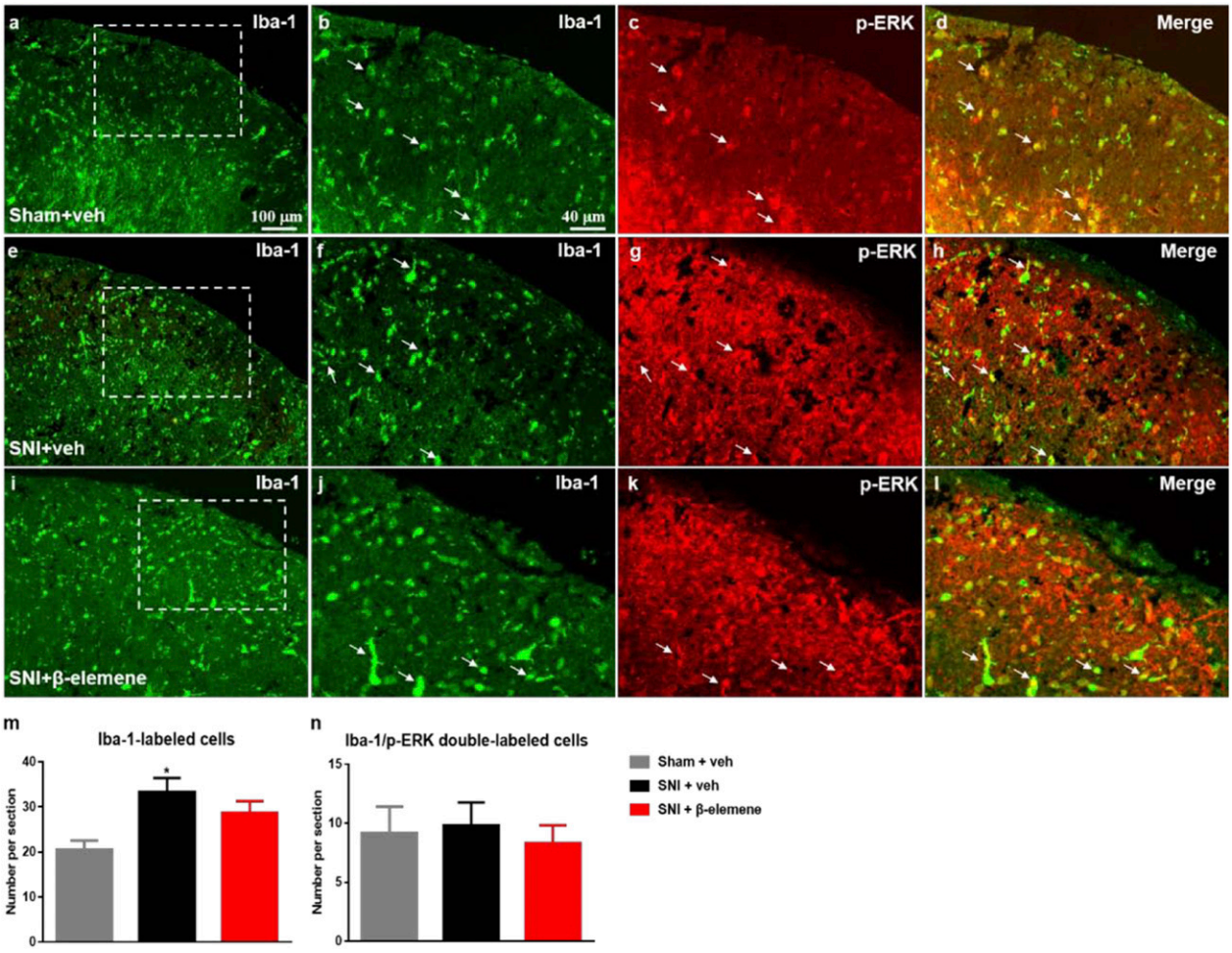

Figure 5. β-elemene exerted no effect on microglial expression of pERK in SNI rats. SNI exerted no effect on the number of Iba-1 or Iba-1/p-ERK double-labelled cells (a-d, e-h) within the ipsilateral spinal dorsal horn on Day 29; β-elemene exerted no effect on the number of Iba-1 or Iba-1/p-ERK double-labelled cells (i–l) in SNI rats. The framed area in images (a, e and i) was magnified in images (b-d, f-h and j-i), respectively. White arrows show double-labelled cells in the images of each set. Bars = 100 μm in images (a, e and i) and 40 μm in images (b-d, f-h and j-l). The numbers of Iba-1 and Iba-1/p-ERK double-labelled cells among the three groups are shown in m and n, respectively (n = 6 sections from three rats in each group).

Then, we examined the effect of β-elemene on spinal JNK activation. First, no change in the number of NeuN-ir cells within the ipsilateral SDH was observed among the three groups (Supplemental Figure 3), suggesting that neuronal apoptosis is not involved in β-elemene analgesia. Second, no change in the number of p-JNK-ir or NeuN/p-JNK double-labelled cells was observed (Supplemental Figure 3). Finally, SNI induced a significant increase in the number of GFAP/p-JNK double-labelled cells, which was reversed by β-elemene (Supplemental Figure 4). Since no change in the expression of p-JNK was observed among these groups, we speculated that the change in the number of GFAP/p-JNK double-labelled cells may be due to the expression change in GFAP-ir cells themselves in different groups. Thus, spinal JNK inhibition may not be involved in β-elemene analgesia.

Finally, the effect of β-elemene on spinal p38 activation was explored. The immunostaining data showed no change in the number of p-p38-ir cells, NeuN/p-p38 double-labelled cells (Supplemental Figure 5) or Iba-1/p-p38 (Supplemental Figure 6) double-labelled cells within the ipsilateral SDH among these groups (Supplemental Figure 5), suggesting that spinal p38 inhibition may not be involved in β-elemene-induced analgesic processes.

β-elemene inhibited SNI-induced upregulation of GFAP and p-ERK in the SDH

Prior immunostaining data indicated that β-elemene inhibited SNI-induced astrocytic activation. One caveat is that GFAP antibodies for immunostaining might recognize solubility or conformation changes instead of actual changes in its expression.[39] Considering these findings, immunoblotting was adopted to validate the results of GFAP immunostaining. Immunoblotting analyses showed that SNI significantly

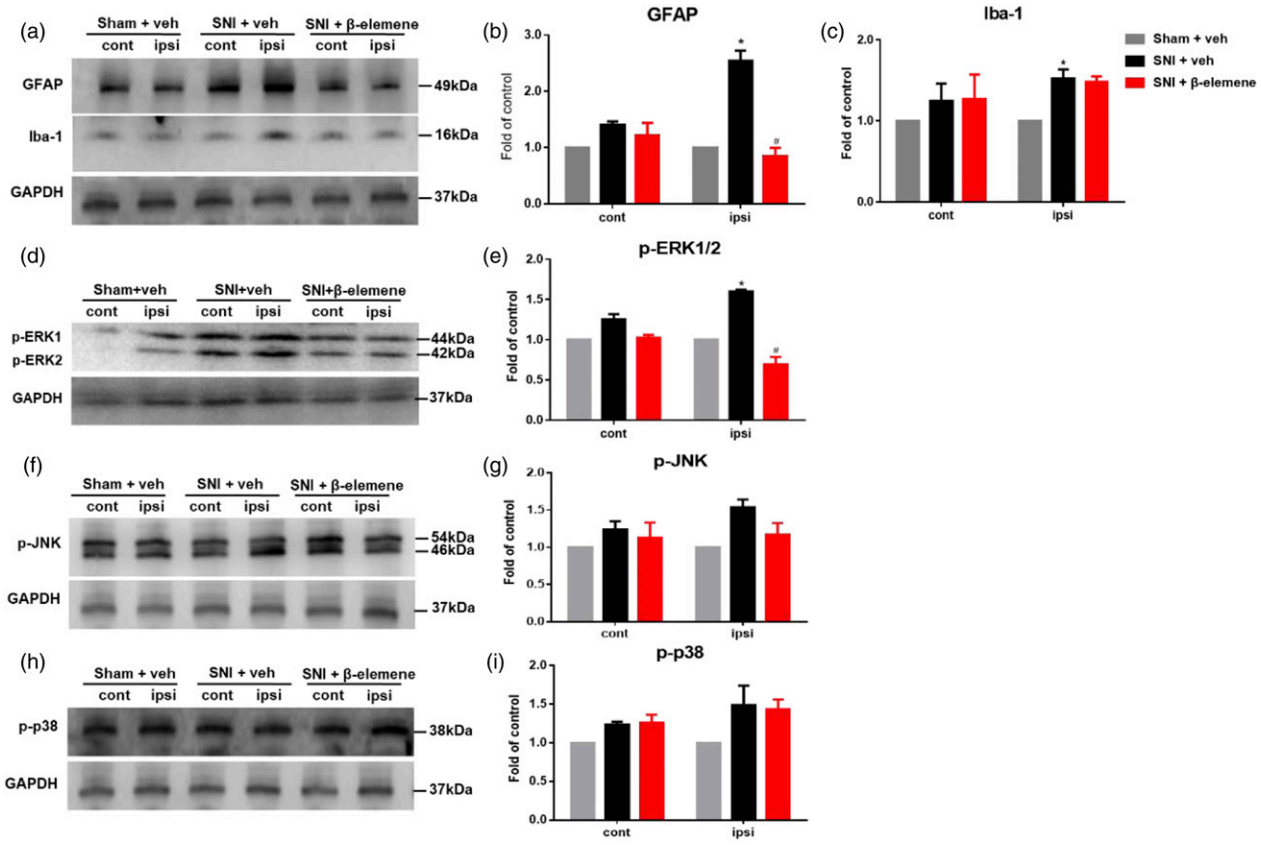

Figure 6. The effects of β-elemene on the expression of GFAP, Iba-1, p-ERK, p-JNK and p-p38 within the spinal dorsal horn in SNI rats on Day 29. Representative western blot samples for each molecular within the spinal dorsal horn among the three groups are shown in a, d, g and h, One-way ANOVA, $n = 3$ rats in each group, $^{*}p < 0.05$, SNI + veh group versus Sham + veh group; $^{\#}p < 0.05$, SNI + β-elemene group versus SNI + veh group.

upregulated the expression of GFAP within the ipsilateral SDH but not the contralateral SDH on Day 29, which was reversed by β-elemene (Figure 6(a)). Moreover, the expression of Iba-1 was robustly upregulated within the ipsilateral SDH in SNI rats. However, no change in the expression of Iba-1 was observed between SNI rats treated with saline and β-elemene (Figure 6(a)). In accordance with the immunostaining results, these immunoblotting data suggest that β-elemene inhibits SNI-induced upregulation of GFAP in the SDH.

Further analyses showed that SNI surgery remarkably upregulated the expression of p-ERK within the ipsilateral SDH, instead of the contralateral SDH, and β-elemene robustly decreased the upregulation of p-ERK in SNI rats (Figure 6(d)). Additionally, there was a trend towards increased expression of p-p38 and p-JNK within the ipsilateral SDH in SNI rats compared to sham rats, and β-elemene did not affect the expression of p-p38 or p-JNK in SNI rats (Figures 6(f) to (h)). In line with the immunostaining data, these results suggest that β-elemene inhibits SNI-induced recruitment of ERK in the SDH.

β-elemene inhibited SNI-induced production of proinflammatory cytokines in the SDH

Astrocyte ERK activation leads to the synthesis and release of proinflammatory factors, which facilitates the development and maintenance of pathological pain after nerve injury.[40] To elucidate the potential anti-inflammatory effect of β-elemene under the conditions of chronic pain, the content of three representative proinflammatory cytokines, including TNF-α, IL-1β and IL-6, within the ipsilateral SDH was measured. Our data showed that SNI surgery robustly enhanced the levels of TNF-α (Figure 7(a), $P < 0.05$), IL-1β (Figure 7(b), $P < 0.05$) and IL-6 (Figure 7(c), $P < 0.05$) compared to the sham group, which was reversed by β-elemene treatment. These data suggest that β-elemene might mitigate neuropathic pain through its antineuroinflammatory effects.

β-elemene inhibited SNI-induced oxidative stress in the SDH

In parallel with neuroinflammation, mitochondrial impairment also plays a crucial role in the progression of

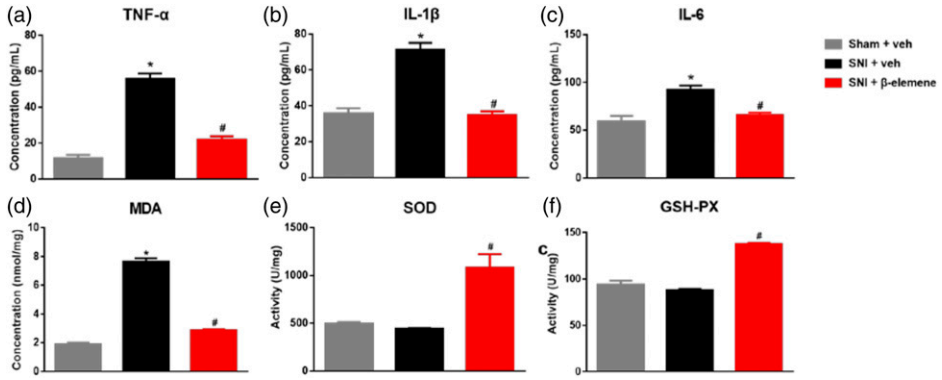

Figure 7. The effects of β-elemene on the content of TNF-α (a), IL-1β (b), IL-6 (c), MDA (d), SOD (e) and GSH-PX (f) within the spinal dorsal horn in SNI rats on Day 29. One-way ANOVA, $n = 3$ rats in each group, $^{*}p < 0.05$, SNI + veh group versus Sham + veh group; $^{\#}p < 0.05$, SNI + β-elemene group versus SNI + veh group.

neuropathic pain.[41] The production of reactive oxygen species (ROS) by spinal glia further exacerbates inflammation and contributes to neuropathic pain.[42,43] To validate the potential antioxidative effect of β-elemene under chronic pain, the contents of MDA (an indicator of membranelipid peroxidation), SOD and GSH-PX (two representative antioxidases) were examined. Our data showed that SNI led to a significant increase in the MDA content within the ipsilateral SDH, which was reversed by β-elemene (Figure 7(d), $P < 0.05$). Although SNI injury did not affect the contents of SOD and GSH-PX, β-elemene increased the levels of SOD and GSH-PX in SNI rats (Figures 7(e) and (f), $P < 0.05$). These data provide evidence that β-elemene might relieve neuropathic pain by augmenting the activity of antioxidant enzymes and mitigating the peroxidation level.

Discussion

β-elemene as a promising medicine for the treatment of chronic pain

Curcuma wenyujin has been a traditional medicinal herb for the management of cardiovascular disease and pain for over one thousand years in China. Emerging evidence suggests that *C. wenyujin* possesses potent antinociceptive, anti-inflammatory, antineoplastic and antiviral activities.[44,45] As the major quality control marker of *C. wenyujin*,[9,10] β-elemene has also been considered to exhibit antitumor and anti-inflammatory effects.[13,16] In the field of pain treatment, β-elemene has only been proven to be effective in cancer pain in Chinese medical practice.[14,15] In the present study, we first characterized the antinociceptive effect of β-elemene in a rat model of SNI-induced neuropathy. We observed that consecutive intraperitoneal injection (*i.p.*) of β-elemene dose-dependently alleviated SNI-induced mechanical hypersensitivity without anti-allodynic tolerance. An interesting phenomenon worth mentioning is that β-elemene exerts delayed analgesic effects (nearly 14 days after initial dosage) in both the early and later phases of neuropathic pain. Such phenomenon has also been reported under the condition of cancer pain in a 1997 clinical study in china.[46] Further studies are needed to explore the possible neurobiological and pharmacological mechanisms underlying this phenomenon. This delayed effects indicated that the combination of β-elemene and rapid-acting analgesics, such as opioids,[47] may bring about better analgesic effects in clinic.

The second advantage of β-elemene in neuropathic pain management is its potential neuroprotective activity, since β-elemene could promote locomotor behavioural recovery by reducing neural apoptosis and enhancing neural regeneration in rats with spinal cord injury.[48,49] Clinical data demonstrated that compared with chemotherapeutic drugs, β-elemene had fewer side effects, including heart, liver, kidney and bone marrow damage.[50] In this study, neither locomotor disturbance nor liver and kidney injury were observed in rats. All these merits suggest that it could be an eligible candidate for chronic pain management. The previous decade has witnessed the blossom of the secondary development of β-elemene. The establishment of novel drug delivery systems improves its bioavailability, and some β-elemene derivatives with stronger pharmacological effects have also been synthesized,[50] which will undoubtedly provide an unprecedented opportunity for the clinical use of β-elemene.

The antineuroinflammatory effect of β-elemene by inhibiting astrocytic ERK activation

Accumulating data based on glial markers indicate that an early spinal microglial reaction leads to a delayed but sustained astrocytic reaction via glial-glial interactions after peripheral nerve injury,[51–53] which plays a key role in the induction and maintenance of persistent pain states, respectively.[19,20] Herein, the upregulation of GFAP and Iba-1 was verified by immunostaining and immunoblotting in SNI rats on Day 29, in accordance with previous studies showing the long-term activation of spinal glia during neuropathic

pain.[54–56] Furthermore, we observed that β-elemene inhibited the expression of GFAP but not Iba-1 in SNI rats, indicating that the spinal astrocytic mechanism may be involved in β-elemene analgesia.

Glial p-ERK expression after nerve injury is highly dynamic. Transient ERK activation in neurons (within hours) drives microglial ERK activation (first week) via neuronal-glial communication in the early phase. Then, astrocytic ERK is recruited by glial-glial interactions in the late phase. The sequential activation of ERK in microglia and astrocytes is important for the initiation and maintenance of neuropathic pain, respectively.[18,57] ERK activation is essential for glial intracellular signalling that leads to the production of proinflammatory cytokines, chemokines and growth factors and then the sensitization of dorsal horn neurons. Drugs aimed at inhibiting ERK activation exhibit desirable analgesic effects.[17,57,58] Herein, we observed that in the late phase of SNI, p-ERK was mainly expressed in astrocytes, and SNI induced obvious ERK activation in astrocytes instead of microglia, further verifying the role of astrocytes in the maintenance of pathological pain. Additionally, astrocytic ERK activation was reversed by β-elemene, suggesting that it may exert analgesic effects through astrocytic ERK pathways.

p-JNK is expressed in spinal astrocytes but not microglia. Nerve injury induces a slow (3 days after operation) but persistent activation of JNK (3 weeks later) in spinal astrocytes,[59,60] which contributes to the maintenance of neuropathic pain via the release of chemokines.[61] Herein, no increase in the expression of p-JNK (albeit a trend of increase in the number of p-JNK-ir cells) was seen 29 days after SNI surgery. This difference may be due to the observation time, since prior data mainly focused on spinal p-JNK expression within 3 weeks after operation.[59,60,62,63] Considering these findings, we speculated that spinal JNK activation may return to normal at this time point. There is controversy as to the expression of p-JNK in neurons. Most studies indicated that p-JNK was not expressed in spinal neurons,[60,61,63] except one reported by Wang XW et al.[64] In line with Wang's result, our data supported that p-JNK was expressed in both spinal astrocytes and neurons.

p-p38 is exclusively expressed in spinal microglia. Nerve injury induces an early (peaking at 3 days after operation), short-term activation of JNK (until 3 weeks later) in spinal microglia,[60,65] which contributes to the induction of neuropathic pain via the release of multiple pronociceptive mediators.[18] In accordance with prior data, we observed that spinal microglial p38 activation returned to baseline 29 days after nerve injury. One major limitation of the present study is the observation time (29 days later), which is past the time window for spinal p38 and JNK activation after nerve injury. Thus, we could not exclude the possibility that the inhibition of glial p38 and JNK activation is involved in β-elemene analgesia. Additionally, β-elemene also modulates the inflammatory response of neutrophils[66] and macrophages,[67] which have been suggested to orchestrate the induction of neuropathic pain within the SDH.[17] Therefore, the effect of β-elemene on these cells and signalling molecules in the early phase of SNI urgently needs to be ascertained.

Astrocytic activation finally leads to the production and release of proinflammatory cytokines, among which TNF-α, IL-1β and IL-6 are the most representative. These astrocytic mediators increase neuronal excitability and synaptic strength via postsynaptic recruitment of AMPARs and NMDARs in dorsal horn neurons through astrocytic-neural interactions.[68] Several lines of evidence have shown that β-elemene can inhibit the inflammatory processes of infectious, autoimmune and traumatic brain diseases by inhibiting the production of proinflammatory cytokines.[21–24] Herein, we observed that β-elemene reversed the upregulation of spinal TNF-α, IL-1β and IL-6 in SNI rats, demonstrating the anti-inflammatory effects of β-elemene under the condition of neuropathic pain. Notably, Gong LY et al. reported that β-elemene could alleviate cancer pain by downregulating spinal NR2B expression.[69] In light of these findings, we propose that β-elemene may indirectly inhibit spinal sensitization by inhibiting astrocyte-related inflammatory processes. As mentioned before, spinal neural activation precedes that of microglia and astrocytes.[57] However, we do not observe the direct effect of β-elemene on neural activation and excitatory synaptic transmission within the SDH, which is a top priority in our future studies.

The antioxidative effect of β-elemene under the condition of neuropathic pain

Apart from neuroinflammation, oxidative stress is another mechanism underlying the generation of neuropathic pain.[70] Oxidative stress results from the imbalance in redox homeostasis, including the overproduction of free radicals together with the overconsumption of the antioxidase system.[71] ROS accumulation not only induces neuronal sensitization directly[70,72] but also facilitates glial activation during neuropathic pain. Activated microglia are the major source of ROS within the SDH upon external stress. After nerve injury, NADPH oxidase (NOX)-derived ROS production is essential for spinal microglial activation and subsequent neuroinflammatory processes,[43,73] forming a positive feedback loop for oxidative stress, since the microglial inhibitor minocycline can also attenuate ROS production during neuropathic pain.[74] The accumulation of ROS further leads to astrocytic activation.[58,75] Accordingly, pharmacotherapies aimed at enhancing the antioxidant system have been reported to effectively alleviate glial MAPK activation and subsequent proinflammatory mediator production during neuropathic pain.[75–79]

The antioxidative role of β-elemene has been explored with respect to cardiovascular diseases. Previous studies have shown that β-elemene remarkably reduced H_2O_2-induced loss in endothelial cell viability via its antioxidative and antiapoptotic activities.[26,80] Moreover, β-elemene inhibited monocyte-endothelial cell interactions by enhancing the antioxidative defence system and suppressing vascular oxidative stress and proinflammatory cytokine production, thus reducing the

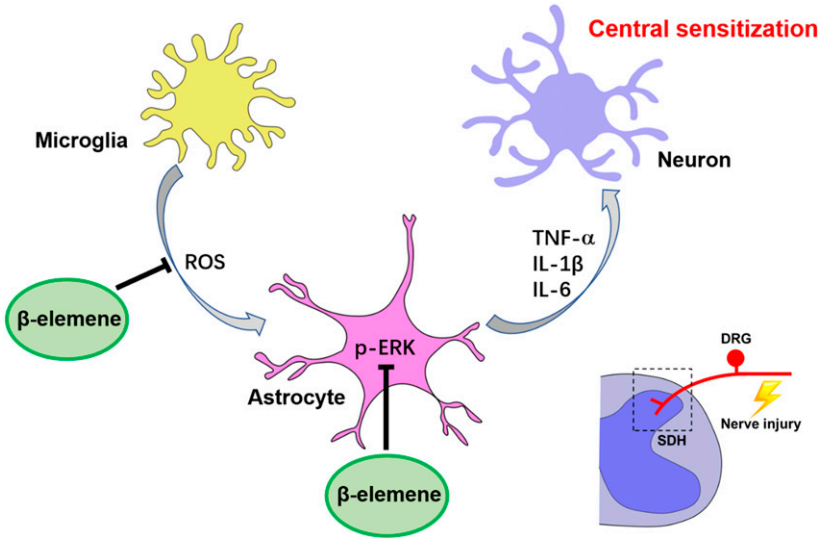

Figure 8. Schematic illustration for the mechanisms underlying the analgesic effect of β-elemene within the spinal dorsal horn. Nerve injury triggers spinal ERK activation and subsequent production of pro-inflammation cytokines, including TNF-α, IL-1β and IL-6, which in turn leads to central sensitization via glial-neural interactions. On one hand, β-elemene could reverse spinal ERK activation and then inhibit neuroinflammation processes. On the other hand, β-elemene could down-regulate the overproduction of reactive oxygen species, which could be presumably derived from spinal microglia, thus indirectly inhibiting the over-activity of dorsal horn astrocytes and neurons. DRG: dorsal root ganglion; p-ERK: phosphorylated extracellular signal-regulated kinase; ROS: reactive oxygen species.

progression of atherosclerosis.[25,81] In the present study, we observed that β-elemene also exerted antioxidative effects during neuropathic pain, as reflected by the enhancement of the antioxidative defence system (SOD and GSH-PX) and the reduction in lipid peroxidation (MDA). In light of these findings, we speculate that the inhibition of free radical production from spinal microglia may be another mechanism by which β-elemene inhibits SNI-induced astrocytic activation.

Conclusions

The results of the present study have demonstrated that pathological activation of astrocytes in the SDH is involved in the late phase of neuropathic pain. β-elemene reversed spinal neuroinflammation processes, possibly by inhibiting astrocytic ERK activation, thus ameliorating neuropathic pain (Figure 8). Additionally, β-elemene exerted antioxidative effects under neuropathic pain. Our findings provide new insights into the analgesic mechanisms of β-elemene via anti-inflammatory and antioxidative actions within the SDH and suggest β-elemene as a novel promising drug for the treatment of chronic pain.

Author contributions

JZ, YQL and JJW designed the study and approved the final version of the manuscript. LTM performed the experiments. YB and PC wrote the manuscript. LTM, KXR and BYF performed the data analysis. JC and TZ participated in some of the immunostaining tests. BYF, HYY and YQ participated in some of the immunoblotting tests. All authors have read and approved the final manuscript.

Declaration of conflicting interests

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Supplemental Material

Supplemental material for this article is available online.

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