

Paeoniflorin attenuates the neuroinflammatory response in a rat model of chronic constriction injury

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Abstract. Neuropathic pain remains the most frequent cause of suffering and disability worldwide. Paeoniflorin (PF), a water-soluble monoterpene glycoside extracted from the roots of *Paeonia lactiflora* Pall, has a wide range of pharmacological functions. Although the neuroprotective effect of PF has been reported in animal models of neuropathology, no systematic investigation has reported on the analgesic properties of PF in neuropathic pain. The aim of the present study was to investigate whether PF can alleviate neuropathic pain and to examine its possible mechanism. Neuropathic pain was induced by chronic constriction injury (CCI) of the sciatic nerve in rats. Following CCI surgery, the rats were administered with PF for 11 days. Mechanical withdrawal threshold and thermal withdrawal latency were assessed prior to surgery, and on days 3, 7 and 11 post-surgery. The levels of interleukin (IL)-1 β and tumor necrosis factor (TNF)- α in the spinal cord were analyzed using enzyme-linked immunosorbent assays. The activation of astrocytes and microglia were observed using immunostaining. In addition, the phosphorylation of p38 mitogen-activated protein kinase (p-p38MAPK) and nuclear factor- κ B (NF- κ B) were examined using western blot analysis. The results indicated that PF significantly attenuated CCI-induced neuropathic pain and decreased the levels of TNF- α and IL-1 β proinflammatory cytokines in the spinal cord. Furthermore, PF inhibited the over-activation of microglia and reduced the elevated expression levels of p-p38 MAPK and NF- κ B in the spinal cord. These results indicated that PF offers potential as a therapeutic agent for neuropathic pain, which merits further investigation.

Introduction

Neuropathic pain is a widespread health problem (1). It is a complex disorder, which leads to chronic illness. Although considerable progress has been made, the mechanisms of neuropathic pain remain to be fully elucidated (2). Accumulating evidence indicates that neuroinflammation may be critical in the initiation and maintenance of neuropathic pain, which is now considered to be a neuroimmune disorder (3-6). Studies have shown that the activation of glial cells, including microglia and astrocytes, contributes to central nervous system neuroinflammation and promotes central sensitization, promoting the subsequent development and maintenance of neuropathic pain (7-9). In addition, several studies have shown that inhibiting microglial and astrocytic activation have analgesic effects in neuropathy (10-12). However, no effective drugs have been found to provide efficient treatment.

Chinese herbs are an important resource for potential novel drugs to develop safe and effective agents for the management of neuropathic pain. Several plants have been found to be effective for antagonizing chronic neuropathic pain (13-15). *Paeoniae alba* Radix, the dried roots of *Paeonia lactiflora* Pallas or *Paeonia veitchii* Lynch, is one of the traditional Chinese crude drug herbs. It has been widely used in traditional Chinese prescriptions to alleviate various issues, such as blood extravasation, blood stagnation and female genital diseases (16). Paeoniflorin (Fig. 1), a monoterpene glycoside, is one of the principal active ingredients of *Paeoniae alba* Radix, and has been reported to exhibit several pharmacological effects, including anti-inflammatory, anti-oxidant and neuroprotective effects (17-20). In nervous disorders in particular, PF has been reported to exert neuroprotective effects against Alzheimer's disease, cerebral ischemia and Parkinson's disease in experimental models (18,21,22). However, no there have been no reports on the analgesic properties of PF in neuropathic pain.

Previous studies have suggested that PF has potent neuroprotective effects by inhibiting inflammatory responses (18,19,23,24). As neuroinflammation is important in the initiation and maintenance of neuropathic pain, the present study hypothesized that PF can ameliorate neuropathic pain. Therefore, the present study aimed to observed the antagonistic effect of PF on neuropathic pain in a rat model

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of chronic constriction injury (CCI) and to examine its underlying mechanism.

Materials and methods

Drugs. PF was extracted from *Paeoniae alba* Radix (Weifang Shengtai Pharmaceutical Industry Co., Ltd., Weifang, China; cat. no. 20150301), and the preparative separation and purification were performed as described previously (25). The purity of PF was determined to be >98% using a high-performance liquid chromatographic assay (Fig. 2). PF was dissolved in normal saline solution (8 mg/ml).

Animals. A total of 30 male Wistar rats (7-week-old; 200–220 g) were obtained from SPF Biotechnology Co., Ltd. (Beijing, China). The rats were housed in a 12-h light/dark schedule at a temperature of $23\pm 2^{\circ}\text{C}$ and a humidity of $60\pm 5\%$ environment with free access to food and water during the 1-week acclimatization period. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The use of the rats was reviewed and approved by the animal care committee of Beijing University of Chinese Medicine (Beijing, China).

CCI of the sciatic nerve. The animals were subjected to CCI, as previously described by Bennett and Xie (26). In brief, the rats were anesthetized with chloral hydrate (300 mg/kg intraperitoneal injection) and the right sciatic nerve was exposed at the mid-thigh level. Proximal to the sciatic trifurcation, adhering tissue was removed from ~ 7 mm of the nerve and four ligatures (chromic catgut 4.0) were tied loosely at 1.0 mm intervals. Sham surgery was performed by exposing the right sciatic nerve without ligation.

Drug treatment. The rats were randomly divided into three groups (10 rats in each group): Sham surgery group (Sham), CCI group (CCI) and CCI+50 mg/kg PF group. The optimal administration dosage of PF was selected according to the results of preliminary experiments. The PF or vehicle (10 ml/kg) was administered by intraperitoneal injections once a day for 11 days, starting on the first day post-CCI.

Mechanical withdrawal threshold assessment. Mechanical allodynia was examined by assessing the paw withdrawal threshold (PWT) in grams using calibrated Von Frey filaments (North Coast Medical, Inc., Gilroy, CA, USA) as described by Chaplan *et al* (27). The rats were placed in transparent plexiglass cages on top of an elevated metal mesh floor, and a series of von Frey filaments of logarithmically incremental stiffness were applied using Chaplan's up-down method at the central region of the plantar surface of the right hindpaw, to identify the filament closest to the pain response threshold. Each measurement was repeated three times at intervals of 15 min, and the average force evoking reliable withdrawal was determined as the threshold. This assessment was performed 1 day prior to CCI surgery, and 3, 7 and 11 days post-CCI surgery.

Thermal withdrawal latency assessment. Thermal hyperalgesia was measured using a BME-410C thermal pain

stimulator (Institute of Medical Biology, Chinese Academy Of Medical Sciences, Beijing, China) as described previously by Hargreaves *et al* (28). The rats were placed in transparent plexiglass cages on top of an elevated glass platform, and appropriate intensity radiant heat ($55\pm 0.5^{\circ}\text{C}$) was applied from underneath the platform to the plantar surface of the hindpaw until the rats showed positive signs of pain (licking or withdrawing the paw). The time taken for the rat to lick or withdraw its paw was recorded and defined as the paw withdrawal latency (PWL). A cut-off time of 25 sec was used to prevent tissue damage. Each measurement was repeated three times at intervals of 15 min, and the average force evoking reliable withdrawal was determined as the threshold. This assessment was performed 1 day prior to CCI surgery, and 1, 3, 7 and 11 days post-CCI surgery.

Enzyme-linked immunosorbent assay (ELISA). On the 11th day following CCI surgery, 60 min following the final drug administration (PF or vehicle), the rats were sacrificed by overdose with chloral hydrate (350 mg/kg intraperitoneal). The L4–L5 spinal cords ipsilateral to the nerve injury were removed, frozen in liquid nitrogen and then stored at -80°C until further processing. The frozen spinal cords were homogenized in cold phosphate-buffered solution (10 μl /mg tissue). Following centrifugation at $10,000 \times g$ for 15 min, the supernatant was used for ELISA. The expression levels of the TNF- α and IL-1 β cytokines were measured using ELISA kits (Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's protocol.

Immunostaining. The L4–L5 spinal cord ipsilateral to the nerve injury was fixed with 4% paraformaldehyde for 24 h and paraffin-embedded and cut into 5 μm thick sections. The spinal cord sections were deparaffinized following routine methods. Sections were then subjected to antigen retrieval (pH 6.0, citric acid antigen repair buffer) and heated in microwave for 15 min (8 min at mid-range and 7 min at low-grade). The sections were blocked using 3% BSA (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 min at room temperature. The sections were then incubated with goat polyclonal anti-ionized calcium-binding adapter molecule-1 (Iba-1) antibody (cat. no. ab5076; 1:100; Abcam, Cambridge UK) or rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; cat. no. BA0056; 1:100; Boster Systems, Inc., Pleasanton, CA, USA) at 4°C overnight. Following extensive washing in PBS, the sections were incubated with appropriate biotinylated secondary antibodies (cat. nos. GB23204 and GB23303; 1:200 in PBS; Goodbio technology Co., Ltd., Wuhan, China) at room temperature for 1 h, followed by incubation using an SABC immunohistochemistry kit (Goodbio Technology Co., Ltd.) according to the manufacturer's protocol. The microglia and astrocytes were stained and observed using an Image-Pro Plus 6.0 imaging analysis system (Media Cybernetics, Inc., Rockville, MD, USA).

Western blot analysis. The protein was extracted by RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology, Haimen, China). Western blot analysis was used to quantify the protein expression of NF- κB p65 in the nucleus and the total protein expression of phosphorylated (p-) p38

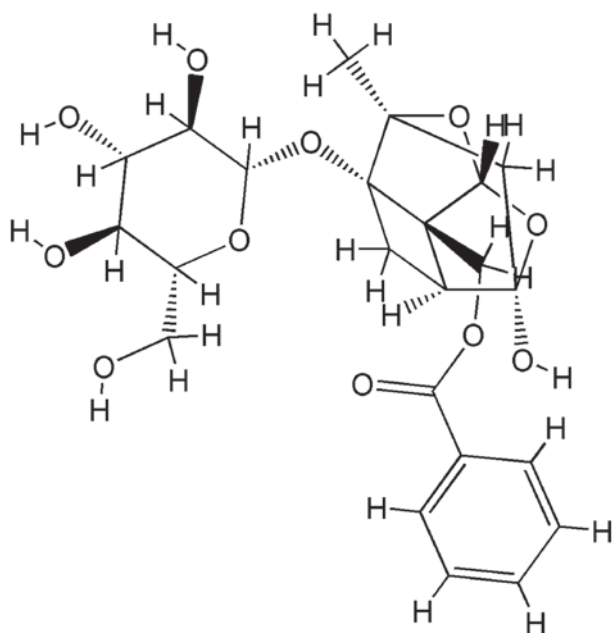


Figure 1. Chemical structure of peoniflorin.

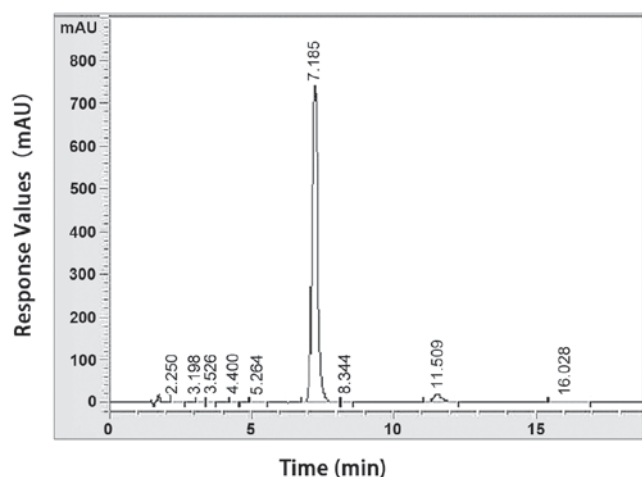


Figure 2. High-performance liquid chromatography chromatogram of peoniflorin.

mitogen-activated protein kinase (MAPK)/p38MAPK in the total protein extracted from the spinal cord. The concentration of the protein was examined by BCA method using protein quantitative kit (cat. no. P0012; Beyotime Institute of Biotechnology). An equal quantity of protein (50 μ g) was loaded and separated by 12% SDS-PAGE. The resolved proteins were transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked in 5% nonfat milk for 2 h at room temperature and incubated overnight at 4°C with mouse anti-NF- κ Bp65 (cat. no. CST6956; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-p-p38MAPK (cat. no. CST4631; 1:1,000; Cell Signaling Technology, Inc.) and rabbit anti-p38MAPK (cat. no. CST8690; 1:1,000; Cell Signaling Technology, Inc.) respectively. The blots were then incubated with goat anti-mouse and goat anti-rabbit secondary antibodies (horseradish peroxidase; Goodbio Technology Co., Ltd.) conjugated with horseradish

peroxidase (1:500, ZSGB-BIO, Beijing, China) for 1 h at room temperature. The protein bands were visualized with chemiluminescence reagent (Engreen Biosystem, Ltd., Beijing, China). Western blot densitometry analysis of signal intensity was performed using Image-Pro Plus imaging analysis system (version, 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. The effect of each treatment was analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean \pm standard error of the mean. Statistical analysis was performed using one-way analysis of variance, followed by the least-significant difference post hoc test or Dunnett's T3 test for comparison of multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PF attenuates increased PWT and PWL in CCI rats. The effects of PF on PWT and PWL in CCI rats are shown in Fig. 3A and B. Prior to CCI surgery, no significant differences were found in the baseline PWL and PWT among all groups. PWT and PWL in the CCI groups decreased markedly 3, 7 and 11 days post-CCI ($P < 0.001$), compared with those in the sham groups, indicating that CCI induced long-lasting thermal hyperalgesia and mechanical allodynia. Following PF administration, the PWL and PWT were significantly increased in the rats, compared with those in the CCI group, on day 11 (PWT; $P < 0.001$; PWL, $P < 0.05$). These results demonstrated that PF produced an antinociceptive effect on CCI-induced pain, including mechanical and thermal hyperalgesia, 11 days following CCI.

Anti-inflammatory effects of PF in CCI rats. To investigate the effects of PF on CCI-induced neuroinflammation, the levels of proinflammatory cytokines, IL-1 β and TNF- α , in spinal cord samples were determined. As shown in Fig. 4A and B, compared with the sham group, the levels of IL-1 β , and TNF- α were markedly increased in the spinal cord of the CCI rats ($P < 0.001$). PF treatment significantly decreased these elevated proinflammatory cytokine levels, compared with the levels in the CCI groups (TNF- α , $P < 0.01$; IL-1 β , $P < 0.05$).

PF inhibits the activation of microglia in CCI rats. To examine the possible mechanisms underlying the protective effects of PF on neuropathic pain in rats, the activation of astrocytes and microglia were monitored. As shown in Fig. 5A-D, compared with the sham group, the expression levels of the astrocyte marker (GFAP) and microglial marker (Iba-1) were increased significantly in the CCI rats ($P < 0.05$). However, compared with the CCI group, the expression of Iba-1 ($P < 0.05$), but not GFAP ($P > 0.05$) decreased significantly in the CCI+PF group, suggesting that PF inhibited the activation of microglia but not astrocytes.

Effects of PF on the activation of p38MAPK in the spinal cord of CCI rats. To further examine the mechanisms underlying the effect of PF, the present study investigated the expression of p-p38 in the spinal cord. As shown in Fig. 6A and B, the expression level of p-p38 was significantly increased in the CCI group, compared with that in the sham group ($P < 0.05$).

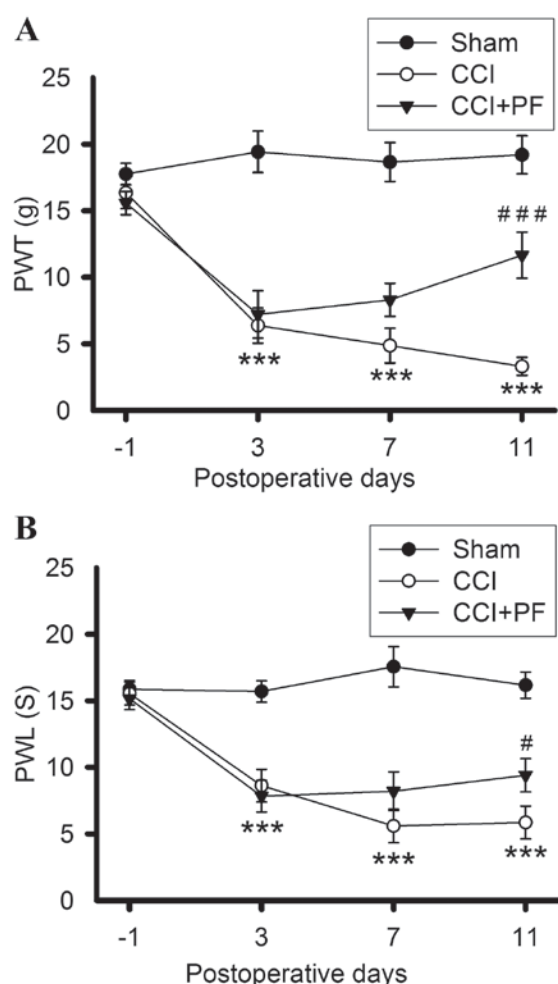


Figure 3. Effects of PF on PWT and PWL in CCI rats. (A) PWT was determined using a von Frey filament test and (B) PWL was measured using a thermal pain stimulator. Data are expressed as the mean \pm standard error of the mean ($n=10$). *** $P<0.001$, compared with the Sham group; # $P<0.05$ and ### $P<0.001$, compared with the CCI group. PWT, paw withdrawal threshold; PWL, paw withdrawal latency; CCI, chronic constriction injury; PF, peoniflorin.

PF treatment markedly decreased the elevated level of p-p38 observed in the CCI group ($P<0.05$). These results demonstrated that PF inhibited the p38MAPK pathway.

Effects of PF on the activation of NF- κ B in the spinal cord of CCI rats. NF- κ B is a crucial transcription factor complex controlling the expression of proinflammatory and pain mediators. To investigate the mechanism underlying the analgesic effect of PF, the present study monitored the expression of NF- κ Bp65, a nuclear protein associated with the NF- κ B signaling pathway, in the spinal cord of the CCI rats. As shown in Fig. 6C, compared with the sham group, the expression level of nuclear NF- κ Bp65 was significantly increased by CCI ($P<0.01$). PF significantly decreased the protein expression of NF- κ Bp65 in the spinal cord, compared with that in the CCI group ($P<0.05$). These results indicated that PF inhibited the NF- κ B pathway.

Discussion

The present study demonstrated for the first time, to the best of our knowledge, that PF alleviated the neuropathic pain induced

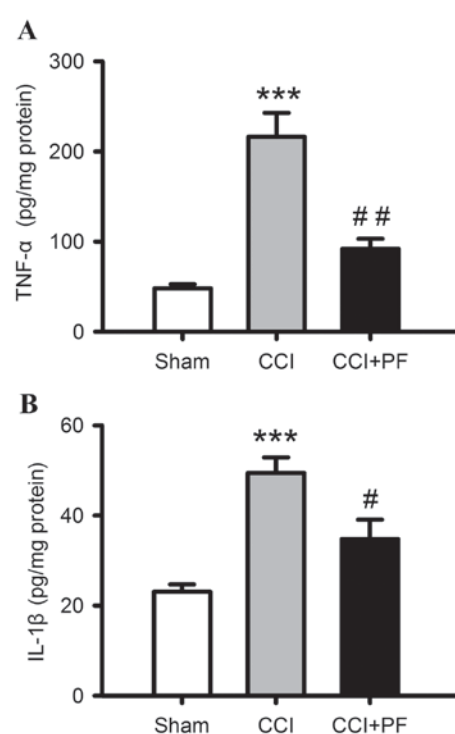


Figure 4. Effects of PF on elevated proinflammation in the spinal cord of CCI rats. Following treatment with PF for 11 days post-CCI, the levels of TNF- α and IL-1 β were measured using an enzyme-linked immunosorbent assay. (A) Expression of TNF- α in the spinal cord. (B) Expression of IL-1 β in the spinal cord. Data assessment was performed in triplicate, ($n=4$). *** $P<0.001$, compared with the Sham group; # $P<0.05$ and ## $P<0.01$, compared with the CCI group. CCI, chronic constriction injury; PF, peoniflorin; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β .

by CCI in rats. It was found that PF attenuated CCI-induced neuropathic pain, including mechanical and thermal hyperalgesia, and decreased expression levels of the TNF- α and IL-1 β proinflammatory cytokines in the spinal cord. In addition, PF inhibited the activation of microglia and reduced the elevated expression of p-p38 MAPK/NF- κ B in the spinal cord induced by CCI. These results suggested that PF offers potential for use as a therapeutic agent for neuropathic pain.

The CCI model is a widely used rodent model to investigate neuropathic pain mechanisms (29-31). This model successfully produces long-lasting thermal hyperalgesia and mechanical allodynia, similar to human behavioral responses (32,33). In the present study, it was found that CCI produced marked mechanical allodynia and thermal hyperalgesia in rats. However, administration of PF for 11 days attenuated mechanical allodynia and thermal hyperalgesia, suggesting the possible therapeutic efficacy of PF. In addition, PF was observed to have a more marked analgesic effect in thermal hyperalgesia, compared with mechanical allodynia. Further investigations are required to examine the underlying mechanism.

Increasing evidence has indicated that proinflammatory cytokines are a critical factor in the initiation and maintenance of hyperalgesia in animal models of neuropathic pain (4). The proinflammatory cytokine-mediated process during neuroinflammation can be induced by nerve injury (5). The CCI model induces the upregulation of proinflammatory cytokines, including IL-1 β , IL-6 and TNF- α , in the spinal cord (34,35). The increase of proinflammatory cytokines in the spinal

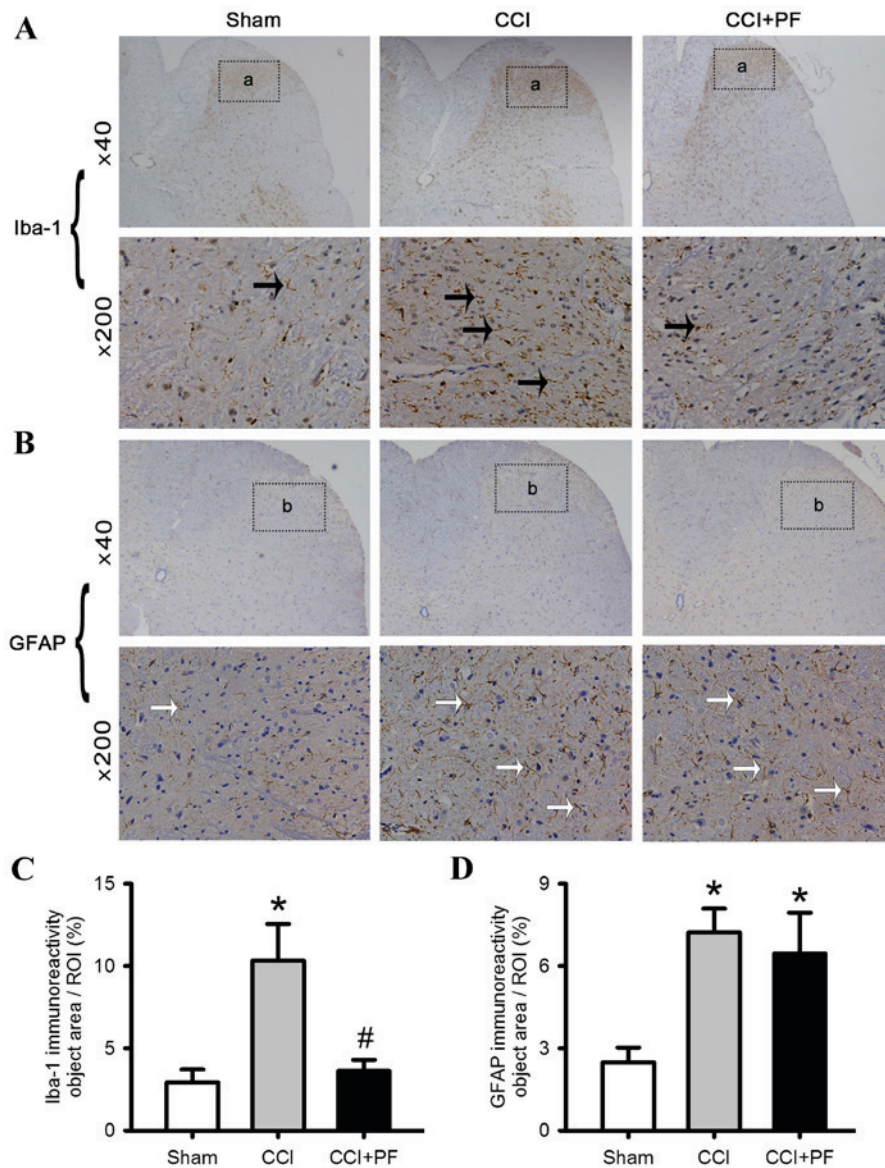


Figure 5. Effects of PF on the activation of microglia and astrocytes in the L4-L5 lumbar segment of CCI rats. Representative images of spinal cord sections stained with astrocyte marker, GFAP, and microglial marker, Iba-1, antibodies. (A) Iba-1 staining in the spinal cord; black arrows indicate Iba-1-positive microglia; (a) regions selected for quantitative analysis of microglial marker activation. (B) GFAP staining in the spinal cord; white arrows indicate GFAP-positive astrocytes; (b) regions selected for quantitative analysis of astrocyte marker activation. (C) Quantification of the effect of PF on the microglial marker Iba-1. (D) Quantification of the effect of PF on the astrocyte marker GFAP. Data are expressed as the mean \pm standard error of the mean ($n=3$). * $P<0.05$, compared with the Sham group; # $P<0.05$, compared with the CCI group. CCI, chronic constriction injury; PF, peanioniflorin; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule-1.

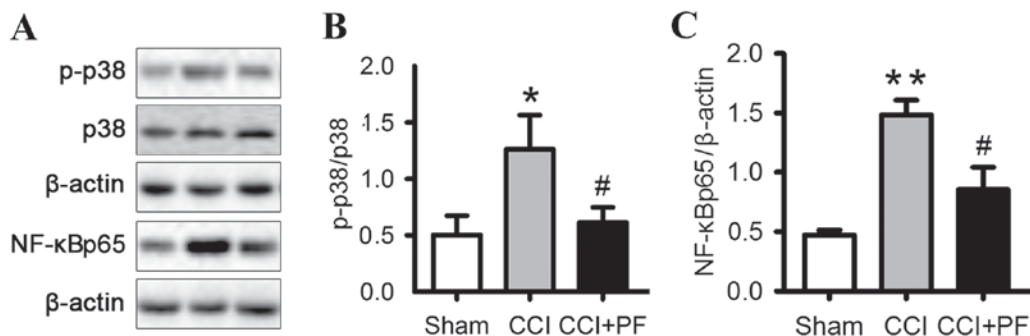


Figure 6. Effects of PF on the expression levels of p-p38 and NF-κBp65 in the spinal cord of CCI rats. Western blot analysis of protein expression levels of p-p38/p38, NF-κBp65/β-actin in the spinal cord 11 days post-CCI. (A) Protein levels of p-p38, p38 and NF-κBp65. (B) Statistical analysis of relative levels of p-p38. (C) Statistical analysis of relative levels of NF-κBp65. Data are expressed as the mean \pm standard error of the mean ($n=3$). * $P<0.05$ and ** $P<0.01$, compared with the Sham group; # $P<0.05$, compared with the CCI group. CCI, chronic constriction injury; PF, peanioniflorin; p-, phosphorylated; NF-κB, nuclear factor-κB.

cord promotes the transduction of detrimental signals by increasing excitatory synaptic transmission and decreasing inhibitory synaptic transmission (36). In the present study, it was found that PF significantly inhibited the overexpression of spinal IL-1 β and TNF- α in the CCI rat model. These results indicated PF as a potential candidate to control neuroinflammation-induced pain.

Following peripheral nerve injury, sensitized primary afferent terminals release nociceptive neurotransmitters and mediators, including glutamate, substance P and fractalkine, which activate spinal microglia and astrocytes (37). Activated microglia and astrocytes contribute to neuroinflammation, accelerate facilitatory pain transmission, and contribute to the subsequent development and maintenance of neuropathic pain (7-9). The results of the present study showed that spinal astroglia and microglia were markedly activated in the CCI injury model, whereas administration of PF for 11 days inhibited the activation of microglia, suggesting that the activities of PF involved regulation of the spinal glial-neuroimmune system.

MAPKs are a crucial molecules in cell signaling, which consist of p38MAPK, extracellular signal-related kinase 1/2 and c-Jun amino terminal kinase 1/2 (38). Emerging evidence has indicates that nerve injury results in the activation of p38MAPK in the spinal cord, and p38MAPK regulates the production of proinflammatory cytokines to promote the development of neuropathic pain (39-41). In addition, several studies have suggested that p38MAPK is critical in microglial signaling under neuropathic pain conditions and represents a valuable therapeutic target for neuropathic pain (42,43). The results of the present study indicated that PF treatment prevented the CCI-induced upregulation in the protein level of p-p38 when measured 11 days post-nerve injury. The same trend was observed in the effect of PF on the inhibition of CCI-induced activation and release of proinflammatory cytokines. These results suggested that PF-mediated inhibition of CCI-induced p38MAPK activation is a possible mechanism underlying its inhibitory action on neuropathic pain.

NF- κ B, a pleiotropic factor, which regulates several physiological processes and is important in regulating the immune response (44). Previous studies have demonstrated that the activation of NF- κ B occurs in the spinal cord, which is involved in the transmission and processing of nociceptive information. Following activation, NF- κ B transfers into the nucleus and regulates the synthesis and release of proinflammatory cytokines, including IL-1 β , IL-6 and TNF- α , which may be crucial in neuroinflammation (45,46). It has been reported that the administration of NF- κ B inhibitors exerts an analgesic effect in various animal pain models (47,48). In the present study, it was found that PF reduced the CCI-elevated expression of NF- κ B in the spinal cord. In addition, PF inhibited the activation of NF- κ B and the subsequent expression of proinflammatory cytokines, which may be beneficial for reducing neuropathic pain.

In conclusion, the results of the present study demonstrated that PF produced a significant analgesic action in CCI-injury rats and that this activity was associated with the modulation of neuroinflammation in the spinal cord. These results suggested that PF is a potential therapeutic agent for neuropathic pain, which merits further investigation.

Acknowledgements

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