

Original Research Article

Vitexin alleviates neuropathic pain in a mouse chronic constriction injury model by inactivation of NF- κ B

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Abstract

Purpose: To investigate the therapeutic effect of vitexin on neuropathic pain (NP) in a mouse model of chronic constriction injury (CCI).

Methods: The CCI model was established by four chronic ligatures in the sciatic nerve. Vitexin was intraperitoneally administered (10 mg/kg, once daily) for 21 days. Mechanical withdrawal threshold (MWT) and paw withdrawal latency (PWL) were determined before and after the establishment of CCI model. The spinal cords were collected to measure mRNA levels by reverse-transcriptase polymerase chain reaction (RT-PCR) enzyme-linked immunosorbent assay (ELISA). Western blot was used to examine protein expression levels.

Results: Vitexin reversed the CCI-induced reduction in MWT and PWL values, indicating that it lowered mechanical hypersensitivity response and hyperalgesia caused thermal stimulation ($p < 0.05$). The elevated levels of IL-6, IL-1 β , and TNF α observed in CCI-treated mice were also inhibited by vitexin, suggesting that it suppressed pro-inflammatory cytokines. Moreover, vitexin attenuated CCI-induced activation of NF- κ B signaling in CCI-treated mice ($p < 0.05$).

Conclusion: Vitexin alleviates NP by inhibiting pro-inflammatory cytokines and NF- κ B signaling in CCI-treated mice. Thus, it is a potential target for NP treatment.

Keywords: Vitexin, Neuropathic pain, Chronic constriction injury, Mechanical hypersensitivity, Hyperalgesia, NF- κ B

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INTRODUCTION

Pain result from a lesion or somatosensory nervous system dysfunction is called as neuropathic pain (NP) [1]. Over the past decades, the incidence of NP has remarkably increased, and about 15-25% of patients with chronic pain have NP [2]. Moreover, NP patients have poorer quality of life than that with

nociceptive pain [3]. The increased occurrence of NP is largely attributed to a lack of effective medications. Therefore, it is necessary to discover effective drugs for NP patients.

Vitexin is a type of flavonoid extracted from the leaves of *Crataegus pinnatifida* (hawthorn) [4]. Previous studies have demonstrated that vitexin exhibits pharmacological effects on tumors and

viruses and in the cardiovascular system and neurons [5-8]. In particular, a recent study reported that vitexin has potency on pain relief by exerting analgesic effects on inflammatory pain through inhibiting TRPV1 and inflammatory responses [9]. Postoperative pain also could be attenuated by vitexin through its effects on opioid and gamma-aminobutyric acid (GABA)_A receptors [10]. However, the potential beneficial effects of vitexin on NP remain unclear.

The NF- κ B signaling pathway is closely related to multiple biological processes through regulating cytokine expression and immune responses [11]. The regulation of inflammatory cytokine gene expression caused by the activated NF- κ B pathway have been proved to affect pain behavior [12]. Through inhibiting NF- κ B signaling, dexmedetomidine relieved NP following chronic constriction injury (CCI) [13]. Considering the conclusion that vitexin could suppress the NF- κ B pathway [14], it is hypothesized that vitexin may ameliorate NP by suppressing NF- κ B activation.

To verify it, a CCI mouse model was established and spinal cords were collected to determine whether vitexin treatment could attenuate mechanical hypersensitivity response and hyperalgesia to thermal stimulation by inhibition of pro-inflammatory cytokine expression and NF- κ B signaling.

EXPERIMENTAL

Animals

C57BL/6 mice (male, 5-week-old) from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China) were housed in specific pathogen-free rooms at $22 \pm 2^\circ\text{C}$ with $60 \pm 5\%$ humidity. Animal experiments were performed according to international guidelines [15], and were approved by the institutional ethics committee (approval no. L20190131).

CCI model

Intraperitoneal chloral hydrate (4 %, 10 $\mu\text{L/g}$) was used for anesthesia. An incision was made in the skin, and about 10 mm of sciatic nerve was gently exposed via blunt scissors, opening a gap between the biceps femoris muscles and gluteus superficialis. Four loose catgut 6.0 ligatures (1 mm apart) were tied near the trifurcation of sciatic nerve. The muscle layer and skin were closed with sutures. Then, the mice were placed onto an electric blanket (37°C) until they regained consciousness.

Mice were grouped (n = 10/group): 1) sham, 2) CCI, and 3) CCI + Vitexin (Vit). CCI + Vit, mice after CCI surgery were intraperitoneally injected with vitexin (10 mg/kg, Tauto Biotechnology Co., Ltd., Shanghai, China) once daily for 21 days. Sham, no ligatures were tied near the trifurcation of sciatic nerve and received normal saline as blank control. CCI, mice after CCI surgery received normal saline as negative control. The spinal cord was collected for following analysis.

Evaluation of MWT and PWL

MWT and PWL were performed 1 day prior to surgery (-1 day) and 1, 7, 14, 21 days after surgery. MWT was assessed with the aid of von Frey filaments. Mice were placed on a wire mesh and acclimatized to surroundings for 45 min. A 0.6 g filament was used to touch the plantar surface of mice hind paw. If the mouse reacted by withdrawing the stimulated hind paw or not, filament thickness was increased or decreased, respectively. This procedure was repeated eight times on each hind paw.

PWL was assessed by Hargreaves method. Mice were placed on a glass surface and acclimatized to surroundings for 45 min. The plantar surface of mice hind paw was exposed to a radiant heat source (with a stimulus cut-off of 18 s). Withdrawal latency was quantified by heat sensitivity, which was recorded three times on each hind paw.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from homogenized tissues, and then reverse-transcribed. RT-qPCR was performed using SYBR Green (Takara, Shiga, Japan) and normalized to GAPDH with the following primers.

Assessment of pro-inflammatory cytokine

Tissues were mechanically homogenized in RIPA buffer at 4°C , and centrifuged. The levels of IL-1 β , IL-6, and TNF α in the supernatant were measured using ELISA kits (R & D Systems, Minneapolis, MN, USA).

Western blotting

The supernatant from spinal cord homogenate was tested for protein concentration by BCA Assay (Beyotime). 30 μg proteins were separated on SDS-PAGE and transferred to membranes (Millipore, Burlington, MA, USA) and incubated with primary antibodies (1:1000) against Toll-like receptor 4 (TLR4), inhibitor of NF- κ B (I κ B α),

phosphorylated (p)-I κ B α , p65, p-p65, and β -actin at 4 °C overnight. The membranes were probed with horseradish peroxidase-labeled secondary antibodies. Protein bands were visualized and analyzed. Antibodies were obtained from Abcam (Cambridge, UK).

Table 1: Sequences of primers

Gene	Primer sequence
TNF α	Forward 5'-CTTCTCATTCTGCTTGTG-3'
	Reverse 5'-ACTTGGTGGTTTGCTACG-3'
IL-1 β	Forward 5'-CCTGGGCTGCCTGATGAGAG-3'
	Reverse 5'-TCCACGGAAAGACACAGGTA-3'
IL-6	Forward 5'-GGCGATCGGATGTTGTGAT-3'
	Reverse 5'-GGACCCAGACAATCGGTTG-3'
GAPDH	Forward 5'-CAATGTGTCCGTCGTGGATCT-3'
	Reverse 5'-GTCCTCAGTGTAGCCCAAGATG-3'

Statistical analysis

All experiments were performed in triplicate, the data expressed as mean \pm SD, and analyzed using ANOVA with SPSS 16.0 software (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significantly.

RESULTS

Vitexin attenuated CCI-induced mechanical hypersensitivity response

To investigate the therapeutic value of vitexin on NP, a CCI mice model was established by four chronic ligatures in the sciatic nerve, MWT value was measured. The significantly decreased MWT value was observed in CCI mice than sham group, indicating marked mechanical hypersensitivity response (Figure 1). However, the reduction of MWT was partially reversed by vitexin from day 1 to 21 (Figure 1, $p < 0.01$). These results demonstrated that vitexin alleviated CCI-induced mechanical hypersensitivity response.

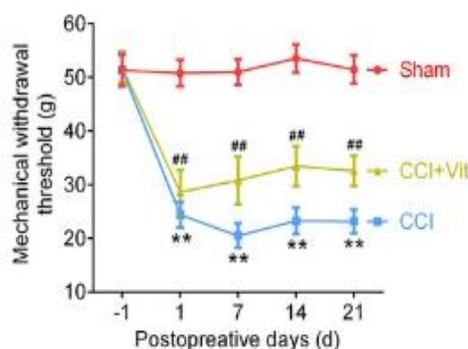


Figure 1: Effect of vitexin on CCI-induced mechanical hypersensitivity response. At 1, 7, 14, 21 days after CCI, MWT value in mice was measured; ** $p < 0.01$ vs. sham/CCI group

Vitexin ameliorated CCI-induced hyperalgesia response to thermal stimulation

Thermal hyperalgesia was also evaluated in the CCI mouse model. The value of PWL was decreased markedly after CCI surgery when compared to sham group (Figure 2, $p < 0.01$). On the other hand, vitexin treatment remarkably attenuated CCI-induced hypersensitivity in mice (Figure 2, $p < 0.01$).

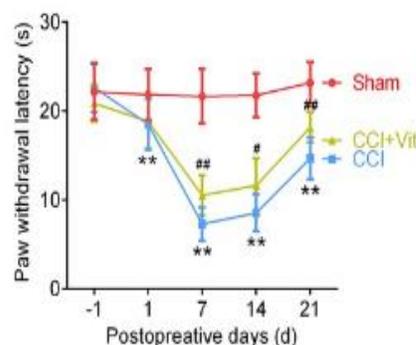


Figure 2: Effect of vitexin on CCI-induced hyperalgesia to thermal stimulation. At 7, 14, 21 days after CCI, the value of PWL was measured; ** $p < 0.01$ and ## $p < 0.01$ vs. sham/CCI group

Vitexin reduced pro-inflammatory cytokine levels in CCI mice

Whether vitexin has anti-inflammatory abilities on CCI mice were evaluated. The mRNA expression and levels of IL-6, IL-1 β , and TNF α were higher in the spinal cord from CCI mice than sham mice. Notably, these levels decreased after vitexin treatment (Figure 3 A and B, $p < 0.01$). These results indicate that the therapeutic effect of vitexin on NP occurs probably by inhibiting pro-inflammatory cytokines.

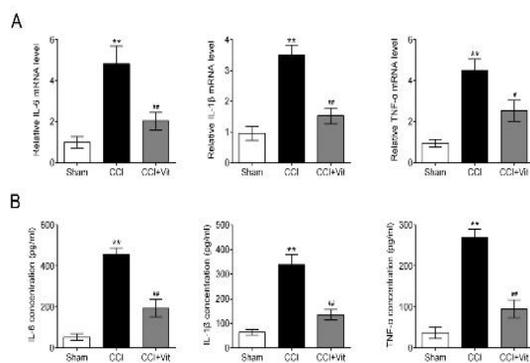


Figure 3: Effect of vitexin on CCI-induced pro-inflammatory cytokine levels in mice. The relative mRNA expression (A) and levels (B) in mice ($n = 5$) were detected by RT-qPCR and ELISA; ** $p < 0.01$ vs. sham/CCI group

Vitexin suppressed NF-κB signaling in CCI mice

Following analysis examined the expression of NF-κB pathway, an essential transduction signal that regulates pro-inflammatory cytokine expression. Treatment of CCI significantly downregulated IκBα, but increased the phosphorylated levels of p65 and IκBα in CCI mice. Vitexin treatment effectively inactivated NF-κB pathway (Figure 4). Thus, vitexin alleviated NP in CCI-mice through inhibition of NF-κB pathway.

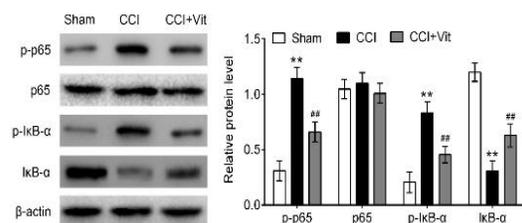


Figure 4: Effect of vitexin on NF-κB pathway in CCI-treated mice; ** $p < 0.01$ vs. sham/CCI group. Protein levels were measured by western blot; ** $p < 0.01$ vs. sham group; ## $p < 0.01$ vs. CCI group

DISCUSSION

Here, vitexin was firstly found to reverse the reductions in CCI-induced MWT and PWL values. Moreover, the enhanced expressions of IL-6, IL-1β, and TNFα in spinal cord induced by CCI was also inhibited by vitexin. Mechanistically, vitexin exerted therapeutic effects on NP through the inactivation of NF-κB signaling.

The common symptoms of NP patients are depression, anxiety, or cognitive deficits [16].

Similarly, cognitive impairment could be induced in a CCI model [17], which is considered an ideal experimental method to establish NP. In the present study, the MWT and PWL were decreased by CCI, suggesting that the model was successfully established [18]. Furthermore, vitexin alleviated MWT and PWL in CCI model, suggesting its potential therapeutic effects on NP.

Following injury to the nervous system, cytokines serve as intercellular messengers [19,20]. Pro-inflammatory cytokines have been found to induce or facilitate NP, while inhibition of pro-inflammatory cytokines alleviates NP in an animal model [21]. Following CCI, TNFα levels were increased in injured nerves, which contributes to the immune response and NP progression [22]. Studies have shown that IL-1β modulates pain sensitivity and contributes to immune responses after nerve injury [13]. Likewise, IL-6 promotes mechanical allodynia following CCI [23]. In this study, vitexin markedly reduced the overexpressions of IL-6, IL-1β, and TNFα in the CCI model. Therefore, the analgesic effect of vitexin on NP was probably mediated through downregulating pro-inflammatory cytokines.

The NF-κB pathway has a crucial role in nociception [24]. In the dorsal root ganglia and spinal cord tissues from multiple animal models of neuropathy, NF-κB signals are activated, thereby contributing to pain development [24]. Inhibitors of NF-κB signaling pathway not only attenuated mechanical hypersensitivity response and hyperalgesia to thermal stimulation in a CCI model but also enhanced analgesia from morphine [25,26]. Vitexin can inhibit NF-κB pathway to show anti-tumor activity in nasopharyngeal carcinoma [14]. In the present study, vitexin significantly inhibited NF-κB signaling in CCI-treated mice, revealing the molecular mechanisms underlying the therapeutic effect of vitexin on NP.

CONCLUSION

Vitexin alleviates NP by decreasing pro-inflammatory cytokine levels and inhibiting NF-κB signaling in the spinal cord. Therefore, vitexin is a potential candidate for NP treatment.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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