Administration of Curcumin Alleviates Neuropathic Pain in a Rat Model of Brachial Plexus Avulsion

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Keywords
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Abstract
Background/Aims: Brachial plexus avulsion (BPA) generally causes a chronic persistent pain that lacks efficacious treatment. Curcumin has been found to possess anti-inflammatory abilities. However, little is known about the mechanisms and effects of curcumin in an animal model of BPA. Methods: Mechanical withdrawal thresholds (MWT) were examined by von Frey filaments. Cold allodynia was tested by the acetone spray test. The levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in rat spinal cords were analyzed by the enzyme-linked immunosorbent assay, and the expression levels of c-Fos and nerve growth factor (NGF) were measured by Western blot. The expression level of glial fibrillary acidic protein (GFAP) was observed by immunofluorescence and Western blot. Results: After curcumin treatment, the MWT showed a significant increase when compared to the BPA group on both hind paws. A remarkable decrease of paw-withdrawal response frequency was observed compared with the BPA group. In addition, curcumin treatment significantly decreased the levels of TNF-α and IL-6 in rat spinal cords that were exceedingly upregulated in the BPA group. The protein levels of c-Fos and NGF were decreased by treatment with curcumin compared with the corresponding protein levels in the BPA group. Besides, curcumin reduced the number of GFAP positive cells and GFAP expression. Conclusions: Our findings suggest that curcumin significantly extenuates the BPA-induced pain and inflammation by reducing the expression level of proinflammatory cytokines and pain-associated proteins and inhibiting the activity of astrocytes.

Introduction
Nowadays, neuropathic pain caused by nerve damage remains a refractory disease due to the lack of efficacious treatment [1–3]. In 2009, Ciaramitaro et al. [4] conducted a multicenter prospective research of the incidence rate of neuropathic pain in patients following traumatic brachial plexus injury. Approximately 56% patients were af-
fected with neuropathic pain among the 107 patients recruited in the study [4]. Moreover, neuropathic pain significantly impaired the quality of life of patients and could even cause depression [5]. Brachial plexus avulsion (BPA) induces intermittent shooting pain and a characteristic persistent oppression, which is usually difficult to cure [6]. The pain could be experienced as a feeling of compression or burning [7]. The mechanisms involved in chronic neuropathic pain include the secretion of inflammatory cytokines, the expression of pain-associated proteins, and the activity of astrocytes [8, 9]. However, the molecular mechanisms of BPA have not been fully understood and still need to be further elucidated [10]. Involving both distinct etiological and pathophysiological factors, pain after BPA has been found to be difficult to treat, as it is resistant to most pain-relieving therapeutics [11].

Curcumin, a constituent of the turmeric plant, has been explored as an efficacious therapeutic drug in preventing and treating various diseases for thousands of years [12]. It has also been found that curcumin possesses a variety of pharmacological and biological properties in drug employment, and has been extensively applied for the treatment of various aging-related diseases, including cancer, atherosclerosis, hypertension, rheumatoid arthritis, cardiovascular diseases, neurodegenerative diseases, ocular diseases, infection and chronic inflammation, hyper tension, and diabetes [13, 14]. In addition, previous findings have demonstrated that curcumin exhibits therapeutic potential on antiviral, antioxidant, antifungal, and anti-inflammatory properties [15]. Besides, toxicity studies proved that it is fairly safe even in high doses to a degree of 12 g in humans [16]. These previous findings indicate a prospective role of curcumin on extenuating the BPA-induced pain and inflammatory responses.

**Materials and Methods**

**Antibodies and Reagents**

Curcumin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 20% dimethyl sulfoxide (DMSO) with 80% normal saline solution. The following antibodies were used: rabbit anti-c-Fos (1:1,500), rabbit anti-nerve growth factor (NGF; 1:600), and mouse anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit anti-glial fibrillary acidic protein (GFAP; 1:500) was purchased from Sigma.

**Animals**

Sprague-Dawley rats (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China), aged 12–16-week-old, were utilized in this study. Rats were placed in plastic cages with a 12-12-hours’ light-dark cycle (dark from 07.00 p.m. to 7.00 a.m.) with excessive food and water supplied. Humidity (54 ± 10%) and room temperature (18–24 °C) were monitored and found to be constant. The animal model of BPA was generated by injecting cobra venom into the lower site of the right brachial plexus. Rats were equivalently and stochastically separated into 3 groups: normal control, BPA (BPA + DMSO), and BPA with curcumin treatment (BPA + Cur). Curcumin was administered at 60 mg/kg body weight (i.p.). The vehicle (20% DMSO solute in normal saline) was applied (i.p.) as controls. Mechanical withdrawal thresholds (MWT) and cold allodynia were examined with von Frey filaments before the experiments and on postoperative days 3, 7, 14, 21, and 28. This study was approved by the Ethics Committee of Quanzhou First Hospital.

**Behavioral Assessments**

Up-down method was applied for examining the MWT. Briefly, rats were housed in a 20 × 20 × 20 cm clear plastic chamber on the surface of a 1.0 × 1.0 cm cell elevated wire mesh. Rats were acclimatized to the environment for 15 min until the spontaneous behaviors ceased before each test. A series of calibrated von Frey hairs (Stoeling, Chicago, IL, USA) with a stiffness of 0.41–15.10 g, with enough force to apply slight bending, hind paws, and hold approximately 6–8 s through the hair perpendicular to the foot surface in the left forelimb were used. When the paws retreat or retreat, positive reactions can be observed. To assess cold allodynia, the acetone spray test was used. For the measurement of cold allodynia, acetone spray test was used as described by Rodrigues-Filho et al. [17]. The rats were housed on a metal mesh covered with a plastic dome, and habituated until exploration reduced. The mid-plantar surface of the hind paw was sprayed with 250 μL of acetone.

**Measurement of Tumor Necrosis Factor-a and Interleukin-6 Levels**

Before getting the tissues for testing, isoflurane was used for anesthetizing the rat deeply. Based on the instructions form the manufacturer, enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) was used for measuring the interleukin (IL)-6 and tumor necrosis factor-a (TNF-a) concentrations in the tissue of lumbar spinal cords. The 450 nm absorbance was measured by an ELISA reader (Bio-Tek, Winooski, VT, USA).

**Western Blot**

The whole protein lysate was quantified using BCA protein assay kit (Haoran Biotech., Shanghai, China). The final protein samples for gel electrophoresis were prepared in SDS-PAGE buffer. The same amount of protein (60 μg) was separated in 10% SDS-PAGE. Polyvinylidene fluoride membranes (IPVH00010, Millipore, Bellerica, MA, USA) was used for electro-transference. Bovine serum albumin was used in the blocking buffer. Primary antibodies, including c-Fos, NGF and GFAP, were used. Imaging was done using an ECL-based system (Santa Cruz Biotechnol., CA, USA). β-actin was used as a loading control.

**Immunofluorescence Characterization of Cells**

To perform the immunofluorescence staining, 4% para-formaldehyde was used for fixing the phosphate-buffered saline (PBS) washed cells. Before using 0.2% Triton X-100 in PBS to permeabilize the cells in room temperature for 20 min, those cells were cleaned by washing 3 times in PBS for 5 min. To block the non-specific binding sites, normal goat serum in PBS was used for 20 min at room temperature. Astrocytes were characterized by incubation overnight with 1:100 diluted rabbit anti-GFAP in humid chamber at 4 °C. In
the same humid chamber, cells were then incubated 2 h with 1:100 diluted TRITC-conjugated goats anti-rabbit IgG at 37 °C. Incubation 5 min with DAPI was performed to stain the nuclei of the cells. After all these steps mentioned above, a fluorescence microscopy (Olympus TH4200) was used to analyze and photograph the fluorescence of the cells. Five different microscopic fields were selected randomly. The number and the percentage of GFAP-positive cells in each different field were calculated.

Statistical Analysis
One- or two-way ANOVA analysis was used to analyze the differences between each sample. When $p < 0.05$, statistical significance can be accepted. All data were shown as the mean ± SD.

Results

Effects of Curcumin on Pain Mechanical Threshold
Animal model of BPA was generated by injecting cobra venom into the lower site of the right brachial plexus. Rats were equivalently and stochastically separated into 3 groups: normal control, BPA (BPA + DMSO), and BPA with curcumin treatment (BPA + Cur). MWT were examined with von Frey filaments before the experiments and on postoperative day 3, 7, 14, 21, and 28. As shown in Figure 1a and b, there were no significant differences in the 3 groups before the operation on both hind paws. Compared with the control group, the MWT on both hind paws of the BPA group exhibited remarkable decreases on postoperative days 3–28. Notably, when treated with curcumin, the MWT showed a significant enhancement when compared to the BPA group on both hind paws. Of note, we did not observe any side effects in the normal animals, including weight loss and death. These results indicate that curcumin might possess an ameliorative effect on BPA in rats.

Effects of Curcumin on Cold Allodynia
Acetone spray test was used for measuring cold allodynia as described by Rodrigues-Filho et al. [17]. As shown in Figure 2a and b, no statistically significant differences were observed before the operation on both hind paws in 3 groups mentioned earlier. All rats introduced to the BPA showed pronounced cold allodynia in comparison to the control group in both hind paws, indicated by the significant enhancement in the paw-withdrawal response frequency on postoperative days 3–28. The administration of curcumin showed a significant attenuation of cold allodynia induced by BPA in both hind paws, with a remarkable decrease of paw-withdrawal response frequency compared with that of the BPA group. Thus, these data suggest that the treatment of curcumin could alter the cold allodynia in rats.

Effects of Curcumin on the Expression of Proinflammatory Cytokines
Recent findings reveal that curcumin possess anti-inflammatory abilities. This might lead to the speculation about the function of curcumin on the secretion of the proinflammatory cytokines. Thus, we examined the lev-
The mechanisms and effects of curcumin in the animal model of BPA have been studied. Curcumin treatment significantly downregulated the levels of TNF-α and IL-6 in rat spinal cords from 3 groups on day 28 post-BPA surgery by ELISA. The results revealed that levels of TNF-α (Fig. 3a) and IL-6 (Fig. 3b) in rat spinal cord that were exceedingly increased in the BPA group were significantly downregulated by curcumin treatment. Since proinflammatory cytokines have been shown to be one of the main causes of BPA, the anti-inflammatory effects of curcumin are precisely one of the mechanisms by which it exerts its analgesic effects.

**Effects of Curcumin on the Expression of c-Fos and NGF in BPA Rats**

Previous reports have demonstrated that various types of nociceptive stimuli could activate c-Fos in the spinal cord. Therefore, c-Fos could be used as a biomarker of pain in the spinal cord. c-Fos is still highly expressed at 28 days postoperatively, indicating that c-Fos is highly correlated with chronic pain. NGF has been shown to play a critical role in the molecular mechanisms of inflammatory regulated disorder and be closely associated with nerve pain. Thus, we examined the protein levels of c-Fos and NGF in 3 groups by Western blot. As shown in Figure 4a and b, the protein levels of c-Fos and NGF were significantly increased in the BPA group, while this tendency was remarkably reversed by treatment with curcumin. Therefore, these data suggest that the pain-relieving effects of curcumin in BPA rats might be due to the inhibitory role of c-Fos and NGF production.

**Effects of Curcumin on the Expression of GFAP in BPA Rats**

The release of astrocytes activates a series of active substances that specifically regulate the transmission of pain. GFAP is a marker of the activity of astrocytes. Thus, we
examined the expression of GFAP by immunofluorescence and Western blot. First, we analyzed the numbers of cells expressing GFAP in 3 groups. As indicated by Figure 5a, astrocyte-specific GFAP (in red) was significantly increased in the BPA group. With the treatment using curcumin, less red staining was observed compared with that in the BPA group. The same conclusion can be reached by counting the GFAP positive cells. Furthermore, curcumin treatment significantly decreased the GFAP protein expression in BPA group (Fig. 5b). Our data conclude that curcumin could significantly reduce the activity of astrocytes, thereby reducing the transmission of pain perception in the central nervous system, which might be another mechanism of the analgesic effect of curcumin in BPA rats.

Discussion

BPA, caused by avulsion in spinal cord root, is a common but severe condition experienced by human beings [18]. Neuropathic pain that resulted from BPA is normally characterized as intermittent shooting pain and constant crushing, which is often intractable [19]. Approximately 83.3% patients undergo neuropathic pain after BPA, which causes an extra burden on the quality of life of patients who were already impaired by sensory, motor, and autonomic deficits [20]. Treatment of neuropathic pain remains a major challenge because it does not respond to most available medications [21]. Even opioids that are normally used as analgesics are commonly considered to have no effect on neuropathic pain [22]. Standard methods applied to treat neuropathic pain are normally used in managing BPA-induced pain, as evidence-based treatment methods specific to BPA are scarce [23]. Therefore, it is crucial to identify novel drug molecules for treating BPA-induced pain.

Curcuma longa (turmeric), belongs to the ginger family, is a rhizomatous herbaceous perennial plant [24]. It is widely used in traditional Chinese medicine for treating symptoms of hypochondriac pain, mental stress, and mania [25]. 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione (curcumin) is the major constituent of curcuma longa, which has found to possess various properties including anti-inflammatory, immunomodulatory, antioxidative, and neuroprotective effects [26]. In addition, curcumin could be used to treat various neurological disorders, such as major depression, tardive dyskinesia, Alzheimer’s disease, and diabetic neuropathy, as it has neuroprotective effects [27, 28]. Recent studies have also demonstrated that curcumin has an anti-nociceptive effect in treating neuropathic pain [29]. These findings suggested a prospective efficacy of curcumin on pain relieving as a natural pigment.

Previous studies have demonstrated that the BPA model in rats causes long-lasting mechanical and cold allodynia [30]. Allodynia, elicited by a stimulus, is a noticeable feature in patients with neuropathic pain [31]. Neuropathic pain associated with BPA normally causes cold allodynia and mechanical allodynia (increased pain sensitivity) [32]. The pain states are generated and maintained by the activation of microglia and astrocytes in spinal cord, which usually lasts 3 months after BPA [33]. Therefore, we could observe that the MWT, cold allodyn-
Mechanisms and Effects of Curcumin in the Animal Model of BPA

...and activation of microglia and astrocytes could prove whether curcumin affects the neuropathic pain. Our results proved that curcumin could significantly reduce the MWT, cold allodynia, and the activity of astrocytes, suggesting that curcumin might possess pain-relieving effects and reduce the transmission of pain perception in the central nervous system.

It is traditionally believed that pain enhancement is caused by the sensitization of neuronal derivatives such as glutamate, substance P, and nitric oxide on dorsal horn spinal cord neurons [34]. However, recent studies have found increasing evidence that spinal glial cells can be closely linked to the generation and maintenance of various enhanced pain states. The mechanism of how spinal glial cells contribute to increase pain states remains unclear. In addition, dynamic reorganization in damaged sensory neurons generates a significant increase in the retrograde axonal transport of various small proteins, such as cytokines and neurotrophins [35]. Recent studies have found that proinflammatory cytokines participate in peripheral nerve changes caused by injury, which seem to highly correlate with the generation and development of neuropathic pain [36]. Upon activation, spinal glial cells were found to secrete proinflammatory cytokines including IL-1β, IL-6, and TNF-α [37]. TNF-α is major regulator of early degenerative alterations during the process of peripheral nerve damage [38]. In addition, it has also been demonstrated that TNF-α could induce ectopic activity, macrophage enrichment, and axonal damage in peripheral nerves [39]. At present, the most obvious evidence for the involvement of proinflammatory cytokines in the increase of pain comes from research of IL-1β and TNF-α in the spinal cord. Stimulated by various pain enhancing manipulations, the mRNA production, protein expression, and the release of IL-1β and TNF-α in spinal cord are significantly upregulated [40]. In addition, it has been reported that the disruption of IL-1β and TNF-α signaling in the spinal cord using IL-1 receptor antagonist and/or TNF-soluble receptor could attenuate pain-promoting effects in different animal models [41]. In contrast to TNF-α, the pain-mediating effects of IL-6 in spinal glial cells are far less clear. Although IL-6 could play a role as a proinflammatory cytokine, in other cases it can act as an anti-inflammatory cytokine instead [42]. Given that IL-6 can exhibit entirely opposite effects under different circumstances, it might not be surprising that IL-6 has been reported for both pain promoting and pain suppressing effects [43]. We have recently demonstrated that

**Fig. 5.** Effects of the intraperitoneal injection of curcumin on the expression of GFAP in BPA rats. a The immunofluorescence images of astrocyte specific GFAP (in red) and corresponding nuclear staining with DAPI (in blue). Scale bar = 20 µm. b Concentrations of GFAP in rat spinal cords were measured by Western blot at day 28 post-BPA surgery. Histogram bars represent mean ± SE. ***p < 0.001 BPA versus control; **p < 0.01 BPA versus BPA + Cur (n = 5 per group). One-way ANOVA with Tukey post-tests. BPA, brachial plexus avulsion; Cur, curcumin; GFAP, glial fibrillary acidic protein.
curcumin could significantly reduce the expression levels of TNF-α and IL-6, suggesting that curcumin might possess anti-inflammatory effects. Curcumin might also impair the level of IL-1β, indicating another mechanism by which it exerts its analgesic effects.

Conclusions

In conclusion, we found that curcumin could significantly reduce the MWT and the paw-withdrawal response frequency. In addition, curcumin treatment remarkably decreased the levels of TNF-α and IL-6 in rat spinal cord and the protein levels of c-Fos and NGF that were exceedingly upregulated in the BPA group. Also, curcumin reduced the number of GFAP positive cells and GFAP protein expression. Thus, our findings suggest that curcumin significantly extenuates the BPA-induced pain and inflammation by suppressing the expression of pro-inflammatory cytokines and pain-associated proteins and inhibiting the activity of astrocytes. These findings provide scientific basis for the application of curcumin in treatment of BPA-induced pain.

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None.

Ethics Statement

This study was approved by the Ethics Committee of Quanzhou First Hospital.

Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

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