INTRODUCTION

Oxaliplatin is the third-generation of platinum derivate, which is widely used in the treatment of a variety of digestive tract cancer.1 Because of its wide application, the side effects of oxaliplatin has been paid more and more attention, the most common one is peripheral neuropathy, the incidence rate of which is 85–95% and there is a dose-limiting toxicity.2 Though chemotherapeutic researches have been carried out aiming at figuring out the mechanism of oxaliplatin-evoked peripheral neurotoxicity, the currently available analgesics can only alleviate the symptom because of the lack of efficacy and unacceptable side effects.3

In recent years, studies demonstrated that cytokines, as the molecules that transmit intercellular signals, are not only involved in the information transfer of algesia, but also in the mediation of pain information in the spinal cord.4 The mechanism of oxaliplatin-induced neuropathic pain has been unclear, and recently oxidative stress has been valued for its contribution to neurotoxicity.5

Nuclear factor-kappa B (NF-κB), as a multipotent transcriptional regulator, plays an important role in cell survival, immunity, inflammation and so on. NF-κB could be activated when cells suffered oxidative stress, following the phosphorylation and entering the nucleus of it, and then up-regulate the expression of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). In conclusion, these findings suggested that curcumin could alleviate oxaliplatin-induced peripheral neuropathic pain; the mechanism might be inhibiting oxidative stress-mediated activation of NF-κB and mitigating neuroinflammation.

Key words  curcumin; oxaliplatin; peripheral neuropathic pain; nuclear factor-kappa B; oxidative stress; inflammation

Curcumin Alleviates Oxaliplatin-Induced Peripheral Neuropathic Pain through Inhibiting Oxidative Stress-Mediated Activation of NF-κB and Mitigating Inflammation

Xuan Zhang, Zhenbiao Guan, Xiaowei Wang, Dazhi Sun, Dan Wang, Yongjin Li, Bei Pei, Min Ye, Jingyu Xu and Xiaqiang Yue

© 2020 The Pharmaceutical Society of Japan
will clarify the efficacy and mechanism of curcumin through oxaliplatin-induced neuropathic pain model rats, and provide an experimental foundation for its clinical application.

MATERIALS AND METHODS

Chemical Reagents  Oxaliplatin was purchased from Ji-angsu Hengrui Pharmaceutical Co., Ltd. (China, catalog number: 170502AF). Fifty milligrams oxaliplatin was dissolved in 5% glucose solution as the stock solution at concentration of 1 mg/mL and stored at 4°C until use. Curcumin (HPLC purity > 98%) was purchased from Shanghai Xingshu Biotechnology Co., Ltd. (China, catalog number: 110781-200613). Malondialdehyde (MDA) (catalog number: A003-1-2), superoxide dismutase (SOD) (catalog number: A001-3-2), glutathione peroxidase (GSH-Px) (catalog number: A005-1-2) and catalase (CAT) (catalog number: A007-1-1) Assay Kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). IL-6 (catalog number: ab234570), TNF-α (catalog number: ab100785), IL-1β (catalog number: ab100768) enzyme-linked immunosorbent assay (ELISA) Kit were purchased from Abcam (Shanghai, China). Anti-NF-κB (catalog number: ab207297) and anti-phosphorylation-NF-κB (catalog number: ab222494) antibodies were purchased from Abcam (Abcam, Cambridge, U.K.). Anti-TNF-α (catalog number: bs-0078R), anti-IL-1β (catalog number: bs-0812R), and anti-IL-6 (catalog number: bs-0781R) antibodies were purchased from Bioss Biotechnology Co., Ltd. (Beijing, China).

Animals and Treatment  Seven-week-old male specific pathogen-free Sprague-Dawley (SD) rats (weighing of 200 ± 10 g) were provided by the Animal Laboratory Center of Chinese Academy of Sciences (Shanghai, China, SCXK2012-0002). The animals were raised in the animal house of The Second Military Medical University under controlled conditions (12h light/dark cycle), temperature (20–25°C) and humidity (40–60%) with food and water.

Establishment of model rats, according to the method of Homles: Oxaliplatin (4 mg/kg body weight) was intraperitoneally injected twice a week and last for four weeks to establish the oxaliplatin-induced peripheral neuropathic pain model rats (Namely on the 1st, 2nd, 8th, 9th, 15th, 16th, 22nd and 23rd days). After administration, the cumulative dose of oxaliplatin in rats was approximately 32 mg/kg.

All sixty SD rats were randomly divided into six groups (n = 10 per group): (1) Negative Control group; (2) Model group; (3) Low-Cur group: low-dose-curcumin + model rats; (4) Middle-Cur group: middle-dose-curcumin + model rats; (5) High-Cur group: high-dose-curcumin + model rats; (6) Negative + High-Cur group: high-dose-curcumin + SD rats. Animals were acclimated for 1 week before the experiments. The negative Control group was intraperitoneally injected with 5% glucose solution; the low-, middle- and high-Cur groups received a daily gavage of curcumin 12.5, 25 and 50 mg/kg body weight, respectively, the Negative + High-Cur group was received 5% glucose solution by intraperitoneally injection and 50 mg/kg body weight curcumin by gavage. The treatment lasted for 28d. The study was approved by the ethics committee of The Second Military Medical University and conducted in accordance with standards of International Society for Pain (IASP). All efforts were made to minimize the suffering of the rats and to reduce the number of animals to be used.

Mechanical Withdrawal Threshold (MWT) Testing  All rats were tested for mechanical withdrawal threshold respectively at day 0, 7, 14, 21 and 28 during the experiment. Rats were placed in a perspex cage with a wire mesh bottom, which allowed full access to the paws. Behavioral accommodation was allowed for approximately 15 min. The area tested was the mid-plantar of hind paw. The paw was touched with a series of von Frey hairs with stiffness of 2.0 g, a larger degree stimulus was given when 2.0 g could not cause a positive reaction and conversely, if the positive reaction occurred, the smaller degree stimulus was given. The positive reaction was defined as the rats raising or licking of their paws after von Frey filament application. Repeated measurements were conducted on each hind paw five times at intervals of 30 s. Three or more positive reactions were regarded as mechanical hyperalgesia. The maximum stimulus (when ≥15 g) was recorded as 15 g.

Cold Allodynia Testing  Testing of cold allodynia was as Flatters’s method, the experimental equipment and pre-operation preparation are the same as above. In brief, with the animals atop the wire mesh floor, a drop (50 µL) of acetone was placed against the centre of the ventral side of the hind paw and a stopwatch was started. In the following 40 s after acetone application, the rats’ withdrawal response was monitored. All tests were conducted on day 0, 7, 14, 21 and 28 during the experiment and repeated measurements were made on each hind paw three times.

Sciatic Nerve Conduction Velocity Testing  After twenty-eight-day curcumin administration, animals were anesthetized with 20% urethane (5 mL/kg body weight, injected intraperitoneally) and separated the sciatic nerve. For electrophysiology studies, stimulation and recording were performed using thin bipolar platinum wire electrodes. Stimulation electrode was placed at sciatic notch where the efferent of sciatic nerve is; recording electrode is located at the ankle joint where sciatic nerve passing. Monopulse square wave with duration of 0.1 ms were used for stimulation, and the stimulation strength was 1.5 times of threshold. Increase the stimulation gradually to detect sciatic nerve conduction velocity. The motor nerve conduction velocity (MCV) and sensory nerve conduction velocity (SCV) were measured by Medlab biological signal acquisition and processing system. The latency from stimulation to the first peak of the compound action potential (CAP) together with the distance between the electrodes was used for the determination of the nerve conduction velocity.

Nissl’s Staining  After administration, rats were deeply anesthetized with 0.45% pentobarbital Sodium (0.1 mL/kg body weight, injected intraperitoneally) and euthanized. L4–L6 spinal cords were collected. Then, the tissue was quickly fixed in 4% paraformaldehyde, followed by paraffin-embedding and sliced into sections of 5 µm in thickness. Finally, the sections were subject to Nissl staining and the morphological changes of tissues and spinal dorsal horn neurons were observed under the light microscope at 400 × magnifications.

Western Blotting  After all the rats were euthanized and their spinal cords collected, protein concentrations were determined by bicinchoninic acid protein assay kit, mixed the protein samples and loading buffer in a ratio of 4:1 and boiled at 95°C for 10 min. Then separated the mixture through electrophoresison 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. After
that, the membranes were blocked in 5% bovine serum albumin (BSA) for 1 h at room temperature and incubated with primary anti-NF-κB (1:1000), anti-phosphorylation-NF-κB (1:1000) and β-actin (1:1000) overnight at 4°C. Then, these membranes were incubated with appropriate secondary antibody for 1 h at room temperature after washing with TBST for 3 times. The membranes were imaged with enhanced chemiluminescence (ECL) reagent and the density was analyzed using Image J software with β-actin as the internal control. Each experiment was repeated for 3 times.

**Immunohistochemistry (HIC) Staining** The paraffin embedded spinal cords were dewaxed, then dehydrated with xylene and gradient concentrations of alcohol. Antigen retrieval was conducted by autoclaving at 120°C for 15 min in citrate buffer, and then sections were incubated with 3% hydrogen peroxide for 10 min to inactivate endogenous catalase. After that, sections were blocked with 10% goat serum and then primary antibodies TNF-α, IL-1β, IL-6 were added forevornight at 4°C. Afterwards, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature and added with the streptavidin in HRP in the end. The expression of TNF-α, IL-1β and IL-6 in spinal cords were observed under light microscopic and analyzed by Image Pro Plus 6.0 software.

**Determination of Oxidative Stress Indicators** The spinal cord homogenate in 10% saline solution was obtained as mentioned above. The enzyme activities of SOD, CAT, GSH-Px and the content of MDA were determined by commercially available kits and the method of measurement was performed according to the manufacturer's instructions.

**ELISA** The spinal cord homogenate in 10% saline solution was obtained as mentioned above. TNFα, IL-6 and IL-1β were analyzed with ELISA Assay Kit according to the protocol of manufacturer.

**Statistical Analysis** Statistical analysis was performed by using SPSS 21.0 and Graphpad Prism 6.0 software. All data were represented as mean and standard deviation (mean ± S.D.). One-way ANOVA and least significance difference post-hoc test were used to evaluate the significant differences between groups. Values of $p < 0.05$ were considered to be statistically significant.

**RESULTS**

**Curcumin Increases the Body Weight in Model Group** There was no significant difference among the groups before curcumin management. And by 7 d after, body weights in model group were significantly decreased than negative control group ($p < 0.05$) and no remarked difference were found between any other two groups. Then compared with model group, significant increase was observed in high- and middle-cur groups 14 and 21 d after administration, respectively ($p < 0.05$) (Fig. 1a).

**Curcumin Increases MWT in Model Group** Five tests were also conducted during the experiment. On the 7th day after initial injection of oxaliplatin, MWT in model group were significantly decreased than negative control group ($p < 0.05$). On the 14th day, compared with model group,
the situation was attenuated in middle- and high-cur group \((p<0.05)\). And by day 21th, compared with low-cur group, MWT in middle- and high-cur group were significantly increased \((p<0.05)\); likewise, the results were also increased in high-cur group than in middle-cur group \((p<0.05)\) (Fig. 1b).

**Curcumin Alleviates the Increase of Paw-Withdrawal Times after Aceton Application in Model Group** As shown in Fig. 1c, no significant alternation was found before and 7d after curcumin management. And 14d later, paw-withdrawal times of cold allodynia in model group was obviously increased compared with negative control group \((p<0.05)\). 21d after initial injection, the evident effects among the model group and curcumin-middle/high-treated model groups were obtained \((p<0.05)\), and dose-dependent alterations showed up until 28d later \((p<0.05)\).

**Curcumin Increases Sciatic Nerve Conduction Velocity in Model Group** Sciatic nerve conduction velocity was testing after curcumin-treatment. It was observed that MCV and SCV were both remarked decreased in model group compared with negative control group \((p<0.01)\). Similarly, dose-dependent effects were found between the model group and curcumin-treated model group \((p<0.01)\) (Fig. 2).

**Curcumin Repairs Injured the Spinal Cord Cells in Model Group** Nissl staining results of each group are as follows. We chose the spinal dorsal horn to analyze the results. Model group: the neuron distribution in this group was disorganized, cells apoptosis occurred and the number of surviving cells was significantly reduced. The number of Nissl bodies was decreased significantly when compared with negative control group \((p<0.05)\), and some of them appeared fragmentation phenomena. The dyeing of Nissl bodies was light and the shape of them was inhomogenous. Curcumin treatment group: the arrangement of neuron was neat, but the intercellular space was widened. And Nissl bodies fragmentation and disappearance occurred in a small number of neurons, the morphology of which was relatively inerratic and dose-dependent effects arose, and there was significant difference among the groups \((p<0.05)\).

---

![Figure 2](image2.png)

**Fig. 2.** Effects of Curcumin Administration on Nerve Conduction Velocity
All data are shown as mean \(\pm\) S.D. \((n=10)\). *: \(p<0.05\); **: \(p<0.01\).

![Figure 3](image3.png)

**Fig. 3.** Nissl Staining of Spinal Dorsal Horn Was Conducted in all Studied Groups (Light Microscope, ×200)
All data are shown as mean \(\pm\) S.D. \((n=10)\). *: \(p<0.05\); **: \(p<0.01\). (Color figure can be accessed in the online version.)
cur group: the neuron was arranged neatly and compactly, and no obvious Nissl body’s fragmentation was observed. There was no significant difference between the two groups (Fig. 3).

Curcumin Increases the Activity of Antioxidant Enzyme in the Spinal Cord of Model Group As shown in Figs. 4a–c, antioxidant levels (SOD, GSH-Px, CAT) were significantly reduced in model group than negative control group ($p < 0.001$); while the reduction were obviously alleviated after curcumin middle/high-dose treatment ($p < 0.05$). However, peroxidation levels (MDA) showed the opposite results ($p < 0.001$) (Fig. 4d), the results suggested that curcumin increased anti-oxidant enzymes and reduced peroxidation to maintain the balance of redox.

Curcumin Decreases the Expression of p-NF-κB/NF-κB

Fig. 4. Effects of Curcumin Administration on Enzyme Activity of SOD, GSH-Px, CAT and MDA

All data are shown as mean ± S.D. ($n = 10$). *: $p < 0.05$; **: $p < 0.01$.

Fig. 5. Effects of Curcumin Administration on the Expression of p-NF-κB/NF-κB ($n = 10$)

*: $p < 0.05$; **: $p < 0.01$. 
in the Spinal Cord of Model Group To clarify the effects of curcumin on NF-κB-activation, we performed Western blotting to detect whether it could regulate the expression of p-NF-κB/NF-κB. The results showed that p-NF-κB/NF-κB was significantly increased in model group when compared with negative control group ($p < 0.01$). After curcumin treatment, compared with model group, the expression of p-NF-κB/NF-κB was remarkably inhibited and all the alternations were statistically significant in low-, middle- and high-Cur group ($p < 0.01$) (Fig. 5).

Curcumin Decreases the Level of Inflammatory Factors in the Spinal Cord of Model Group By immunohistochemistry staining to determine the expression of inflammatory factors, we chose the spinal dorsal horn and concluded that oxaliplatin injection caused the significantly high level TNF-α, IL-6 and IL-1β ($p < 0.01$). Their expressions were significantly reduced after curcumin administration in all three different doses groups compared with model group ($p < 0.01$) (Figs. 6a–c). Similarly, the ELISA results of TNF-α, IL-6 and IL-1β expression were also consistent with the immunohistochemical results (Figs. 6d–f).

No significant differences were found between negative and Negative + High-Cur group in all the above tests.

DISCUSSION

Oxaliplatin, the specific first-line clinical agents for advanced colorectal cancer, is prone to induce dose-limiting
painful neuropathy\textsuperscript{21}) and the symptoms last for at least 2 years after cessation of drug treatment. The course of treatment and the quality of patients’ life have been greatly affected.\textsuperscript{22)} In recent years, researchers have attached more and more importance to oxidative stress because of its role in the delayed- and accumulated-type of neurotoxicity.\textsuperscript{23)} Mammalian nerves are known to be more susceptible to oxidative stress because of their high content of phospholipids, mitochondria rich axoplasm and also due to weak cellular antioxidant defense.\textsuperscript{24)} In addition, lacking of blood–brain-barrier makes peripheral nervous system more susceptible to the injury of chemotherapeutic accumulation like oxaliplatin, increasing the level of reactive oxygen species (ROS) and destroying the antioxidant-related proteins, and results in the disorder of redox balance in the end.\textsuperscript{25,26)} Moreover, the increase of ROS could attack the unsaturated fatty acids of nerve cells, then significant high levels of lipid peroxidation arise, resulting in the production of large amounts of MDA, so the level of MDA could be used as an indirect indicator of nerve cells’ peroxidation.\textsuperscript{27)} While activation of antioxidant enzymes (SOD, GSH-Px and CAT) could reflect the elimination of ROS to a certain extent,\textsuperscript{28–30} Liu and colleagues\textsuperscript{31)} and his team found that applied antioxidant enzymes early could alleviate dysfunction of neurons, further confirmed the important role that oxidative stress playing in oxaliplatin-induced neuropathic pain.

NF-κB, a transcription factor involved in the genesis and amplification of inflammatory insults at various tissue sites, could be activated by oxidative stress and cause the forming of p-NF-κB. And after phosphorylation, NF-κB could translocate to nucleus and bind with corresponding sequence of the target gene like pro-inflammatory factors and then up-regulate the expression of TNF-α, IL-1β, IL-6 and the like. Many experimental researches in recent years have provide evidence that inflammatory mediators induce or increase neuropathic pain,\textsuperscript{32)} while the key step of which is activation of NF-κB pathway.\textsuperscript{33)} Recently, evidence has emerged that the expression of TNF-α and IL-1β is increased in varieties of neuropathic pain models such as chronic sciatic nerve injury and oxaliplatin-induced peripheral neuropathic pain.\textsuperscript{34–36)} Peripheral or intrathecal application of TNF-α and IL-1β antagonists could effectively inhibit the pain.\textsuperscript{37)}

Curcumin, as a phytochemical isolated from turmeric, has been investigated with promising anti-carcinogenic activity by in vitro and pre-clinical researches, and studies suggested that curcumin is able to target a range of tumorigenic pathways.\textsuperscript{38)} The encouraging clinical results have already shown up of curcumin administration in colorectal cancer.\textsuperscript{39)} Since the meaningful clinical applications in digestive tract cancer of oxaliplatin and its unavoidable to induce neurotoxicity, it is of great significance to explore the effect and mechanism of curcumin in this process.

This study established the oxaliplatin-induced neuropathic pain model rats and the chemotherapeutic agent was suggested to influence the mechanical withdrawal threshold and cold allodynia, and showed the same response with patients.\textsuperscript{40)} Evaluating the function of sciatic nerve is of great importance to know the severity of nerve injury. In our results, oxaliplatin disturbed the sciatic nerve conduction velocity, while curcumin treatment significantly increased MCV and SCV and improved the peripheral nerve function.

Previous studies reported that inflammation and oxidative stress are responsible for the pathophysiological alternations of neuropathic pain.\textsuperscript{41)} The capacity of curcumin to augment the activity of antioxidant enzymes (SOD, GSH-Px and CAT) and mitigate the peroxidation level (MDA) was verified and that is consistent with previous researches.\textsuperscript{42)} Moreover, in agreement with the previous evidences,\textsuperscript{43)} curcumin remarkably inhibited the activation of NF-κB and decreased the levels of pro-inflammatory factors (TNF-α, IL-1β, IL-6). These alternations prove our hypothesis which is, curcumin may alleviate the oxaliplatin-induced neuropathic pain by suppressing oxidative stress, inhibiting the activation of NF-κB, down-regulating expression of inflammatory factors and mitigating neuroinflammation.

In summary, our study identifies curcumin as a potential treatment for alleviate oxaliplatin-induced peripheral neuropathic pain. The mechanism might be inhibiting oxidative stress-mediated activation of NF-κB, down-regulating expression of inflammatory factors and mitigating neuroinflammation. Further studies are needed to clarify its deeper mechanism and clinical utilization.

CONCLUSION

Curcumin could alleviate oxaliplatin-induced peripheral neuropathic pain; the mechanism might be inhibiting oxidative stress-mediated activation of NF-κB and mitigating neuroinflammation.

Acknowledgments This study was supported by funding of the National Natural Science Foundation of China (Grant No. 81302933), and Science and Technology Commission Guidance Project of Shanghai (Grant No. 14401931300, 16401933700).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

8) Hosseini A, Hosseinzadeh H. Antidotal or protective effects of cur-


