

Original Research

Apigenin Alleviates Allodynia and Hyperalgesia in a Mouse Model of Chemotherapy-Induced Peripheral Neuropathy via Regulating Microglia Activation and Polarization

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Abstract

Background: Apigenin has been reported to exhibit anti-inflammatory and anti-oxidative activities. This study aimed to investigate the protective role of Apigenin on chemotherapy-induced peripheral neuropathy (CIPN). **Methods:** CIPN mouse model was established using Paclitaxel treatment. Hot plate and tail prick latency tests were performed to examine the allodynia and hyperalgesia behaviors. Anti-inflammatory and anti-oxidative effects of Apigenin on CIPN were determined by enzyme-linked immunosorbent (ELISA) assay, Western blot, and qRT-PCR. Nuclear recruitment of nuclear factor erythroid 2-related factor 2 (NRF2) was analyzed to evaluate the underlying mechanisms of the protective effects of Apigenin. **Results:** Apigenin significantly alleviated CIPN-induced nociceptive behaviors of CIPN mice. It also decreased the TNF- α and IL-1 β levels, suppressed oxidative stress and inflammation in the surgical spinal cord tissues. Mechanistically, Apigenin altered the pro-inflammatory and anti-inflammatory phenotypes ratio of microglia through promoting the nuclear recruitment of NRF2 and activating the NRF2/Antioxidant Response Element (ARE) signaling pathway. **Conclusions:** In summary, Apigenin relieves CIPN by regulating microglia activation and polarization, which provides a potential therapeutic strategy for CIPN treatment.

Keywords: Apigenin; CIPN; microglia; inflammation; NRF2

1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is one of the most common side effects of chemotherapy drugs, which results in a variety of symptoms including pain, hyperalgesia, tingling, losing sensation, numbness, paresthesia and gait disturbance [1,2]. CIPN affects the life quality of patients and jeopardizes the patient's survival [1]. CIPN is neuropathic pain that caused by axonal degeneration. Multiple agents, including glutathione, amifostine, and vitamin E, were evaluated as prevention strategies for CIPN, with no agent demonstrating efficacy. Moreover, adjunct therapy with topical agents, tricyclic antidepressants, and anticonvulsants, such as pregabalin and gabapentin, have shown limited efficacy [3–5], which suggests that the mechanism of CIPN is different from the typical neuropathic pain. Although American Society of Clinical Oncology (ASCO) recommends duloxetine as the only potential treatment for CIPN [6], unfortunately, several side effects of duloxetine have been reported, including nausea, fatigue, dry mouth, drowsiness, constipation, losing appetite, and dizziness. Furthermore, other drugs approved and licensed for neuropathic pain have been reported have a broad range of debilitating side effects [7]. In addition, traditional analgesics, such as non-steroidal anti-inflammatory drugs and opioids, are clinically ineffective in reducing neuropathic pain, while opioids have addictive side effects, and lead to seizures and respiratory disturbances [8].

Microglia are important neural-specific immune cells that influence brain development, maintenance of the neural environment, and response to neural injury that associated with the development of neuropathic pain [9]. Previous studies have shown that microglia exacerbated neuropathic pain through triggering receptor expressed on myeloid cells 2/DNAX-activating protein of 12 kDa (TREM2/DAP12) signaling [10]. Neurons interact with microglia through purinergic signaling during neuropathic pain [11]. Astrocytes interference with microglia can lead to neuropathic pain by regulating the stromal cell-derived factor 1/C-X-C motif chemokine receptor 4 (the CXCL12/CXCR4) axis [12]. Xu *et al.* [13] demonstrated that nicotinamideadenine-dinucleotide phosphate oxidase (NOX2)-induced oxidative stress can increase the plasma membrane translocation of protein kinase C ϵ (PKC ϵ) and promote neuropathic pain in rat model. Accordingly, inhibition of oxidative stress through applying the mesoporous polydopamine nanoparticles attenuated neuropathic pain in rats [14]. Recent study showed that estrogen reduced neuropathic pain by inhibiting the activation of microglia and astrocytes [15]. Although multiple cytokines and signaling pathways have been identified, the exact mechanism of microglia in neuropathic pain is unclear.

Apigenin belongs to Apigenin family that presents in fruits and vegetables, which has been used as an herbal medicine in China [16]. In mice, Apigenin inhibited the



BV-2 microglia proliferation, resulting in apoptosis [17]. In addition, Apigenin also suppressed the production of prostaglandin E2 (PGE2) and nitric oxide (NO) in BV2 microglia via inhibiting NO synthase type 2 (NOS2) and cyclooxygenase type 2 (COX2) protein levels, and oral Apigenin had protective effects on neuronal injury in mice with cerebral artery occlusion [18]. In view of the regulatory effects of Apigenin on microglia function and neuropathic pain, we explored whether Apigenin can alleviate neuropathic pain by regulating the function of microglia in this study.

2. Materials and Methods

2.1 Animals and Treatment

Eight-week-old CD-1 mice (male) were obtained from GemPharmatech (Nanjing, China). All mice were housed in polypropylene cages under 12–12 hour light-dark cycles at 24 °C with 70% humidity. Mice were fed with standard chow diet and sterile filtered water. The animal study and treatment protocols were approved by Quanzhou First Hospital Affiliated to Fujian Medical University. The CIPN mouse model was established as described previously [19]. Mice were divided into six different groups randomly. One group of mice was intraperitoneally (i.p.) injected with saline, which was set as normal control (NC). The mice in CIPN groups were injected 2 mg/kg/day Paclitaxel (PTX, #T7191, Sigma, St. Louis, MO, USA) from Day 0 to Day 6. The daily administration of Apigenin (#10798, Sigma, St. Louis, MO, USA) was started at Day 4. Three doses (25, 50, 75 mg/kg) were used in this study. The treatment ended at Day 20. Gabapentin (GABA, #1287303, Sigma, St. Louis, MO, USA) injection (100 mg/kg) was set as positive control. The pain behaviors of treated mice were monitored during the treatment, and surgical spinal cord tissues were collected after the treatment for the following analysis.

2.2 Hot Plate and Tail Prick Latency Tests

The basal pain behaviors of the normal control and CIPN mice related to allodynia and hyperalgesia were assessed by hot plate and tail prick latency tests. Hot plate test was carried out to measure the response latencies by using the Perspex cylinder as described previously [20,21]. Tail flick test was carried out to assess the anti-nociceptive effect of certain treatment through detecting the response latencies. The plantar test device (7370 plantar test) was used to performed tail flick test as described previously [22,23]. Tests were carried out on Day 0 (started PTX administration), Day 6, Day 11, and Day 18.

2.3 Spinal Cord Isolation

Mice were euthanized using CO₂ and spinal cord was isolated through hydraulic extrusion method. In brief, the fur was cut open along the spinal column in a distal direction. The spinal column was isolated by cutting on both

sides along the spinal column past the pelvic bone. Then, the spinal column was straightened. Phosphate-buffered saline was inserted into the canal through a 18G cannula. The isolated spinal tissue was homogenized using liquid nitrogen.

2.4 Inflammatory and Antioxidant Profiling

The inflammatory factors (TNF- α and IL-1 β) and oxidative stress-related parameters (malondialdehyde (MDA), oxidized glutathione (GSSG), ratio of glutathione (GSH) and GSSG) in surgical spinal cord tissues were determined using the commercial kits ordered from Beyotime Biotechnology (Shanghai, China) following the protocols provided by the manufacturer.

2.5 Quantitative Reverse Transcription PCR (RT-qPCR)

Total RNA was extracted from the surgical spinal cord tissues, and isolated with Beyotime RNAeasy™ Kit (Beyotime, Shanghai, China). The cDNA was synthesized using the Vazyme Script RT SuperMix (Vazyme, Nanjing, China). ABI StepOne™ System (Applied Biosystems, Foster City, CA, USA) was used to perform qRT-PCR with Vazyme SYBR Green Master Mix (Vazyme, Nanjing, China). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of target genes.

2.6 Western Blot

Total protein of surgical spinal cord tissue was extracted using RIPA buffer containing phosphatase/protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Nuclear and cytoplasmic protein was extracted by using the nuclear extract kit (Active Motif, Shanghai, China). The 12% SDS-PAGE gel and nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) were used for Western blot as described previously [24]. Primary antibodies of CD11b (ab133357, 1:1000 dilution in blocking buffer (5% non-fat milk)), CD68 (ab283654, 1:1000 dilution), and CD206 (ab64693, 1:1000 dilution) were purchased from Abcam (Cambridge, MA, USA). Primary antibodies of NRF2 (#12721, 1:1000 dilution), HDAC1 (#34589, 1:1500 dilution), α -Tubulin (#2125, 1:3000 dilution), and GAPDH (#5174, 1:1500 dilution) were ordered from Cell Signaling (Danvers, MA, USA). The corresponding second antibodies, rabbit (611-005-215, 1:2000 dilution) and goat (805-005-180, 1:2000 dilution), were purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA).

2.7 Statistical Analysis

The Prism 8.0 (GraphPad, San Diego, CA, USA) was used to assess significant differences among different treated groups in this study. Results were shown as mean $\pm$ standard deviation (SD). Differences between treatments were analyzed by a one-way or two-way analysis of variance (ANOVA) followed by a post hoc test.

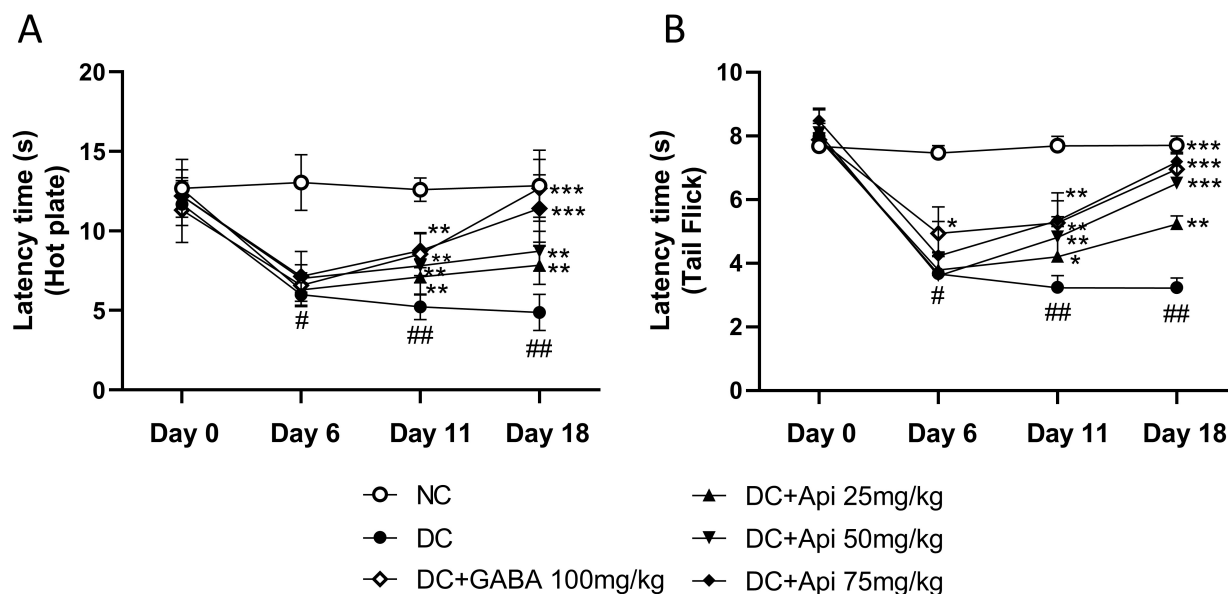


Fig. 1. Apigenin alleviated CIPN-induced nociceptive behavior of CIPN mice. Nociceptive behavior was determined by the latency time measured by hot plate (A) and Tail Flick (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1 Apigenin Elevated Pain Threshold in CIPN Mice

To investigate the therapeutic effects of Apigenin on chemotherapy-induced pain, we established the CIPN mouse model using the PTX treatment as described previously [8], and then performed hot plate and tail prick latency tests. As shown in Fig. 1A, CIPN reduced latency to half in all treated groups except the NC group which did not receive PTX chemotherapy at Day 6. Latency increased significantly in the CIPN-treated groups over time. At Day 11, the latency of GABA group (as a positive control) and Apigenin treated group increased significantly compared to that of disease control (DC) group (Fig. 1A). At Day 18, the latency of high dose of Apigenin treated group significantly increased compared to DC group. The group receiving the medium dose of Apigenin had increase in latency during this period too, which indicated the dose-dependent effect of Apigenin administration in hot plate test. To further assess the modulation of pain sensation and relief of mechanical hyperalgesia by Apigenin treatment, we performed tail prick latency test, and similar results to hot plate test were observed (Fig. 1B).

3.2 Apigenin Inhibited the Production of Inflammatory Cytokines in CIPN Mice

Next, we examined the effects of Apigenin treatment on the levels of the pro-inflammatory cytokines $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ in the spinal cord of control and CIPN mice. Compared with the NC group, on Day 7, the $\text{TNF-}\alpha$ level of DC mice was significantly higher, while $\text{TNF-}\alpha$ level was significantly decreased in GABA and high Apigenin treated groups (Fig. 2A). Interestingly, Apigenin treatment decreased $\text{TNF-}\alpha$ level in a dose-dependent pattern, which

were all statistically significant with three doses and were more obvious on Day 14 (Fig. 2A). Similar trend of $\text{IL-1}\beta$ level was obtained after Apigenin treatment (Fig. 2B). These results indicated that Apigenin reduced the production of inflammatory cytokines in CIPN mice.

3.3 Apigenin Reduced CIPN-Induced Oxidative Stress

The elevated oxidative stress is another important feature of CIPN [25]. Therefore, we examined GSSG and MDA levels and GSH:GSSG ratio in surgical spinal cord tissue to assess the effects of Apigenin treatment on oxidative stress in CIPN mice. In comparison with the control group, the lipid peroxidation in the CIPN group was enhanced, and the level of MDA was significantly increased, while GABA and Apigenin treatments significantly decreased MDA levels in each treatment group (Fig. 3A). Interestingly, GSSG level was significantly increased in CIPN group compared that of the control group. GABA treatment showed a slight decrease in GSSG levels, however, GSSG levels were significantly decreased in all Apigenin treated groups compared to DC group (Fig. 3B). Likewise, GSH:GSSG ratio was significantly elevated in all treatment groups compared to the DC group (Fig. 3C). The above data indicated that Apigenin alleviated CIPN-induced oxidative stress of CIPN mice.

3.4 Apigenin Improved Pro-Inflammatory and Anti-Inflammatory Status in CIPN Mouse

To further explore the underlying mechanism of Apigenin on CIPN, we assessed changes in pro-/anti-inflammatory phenotypes of microglia from protein and gene levels through detecting the expression of microglia markers in the surgical spinal cord tissue. CIPN pro-

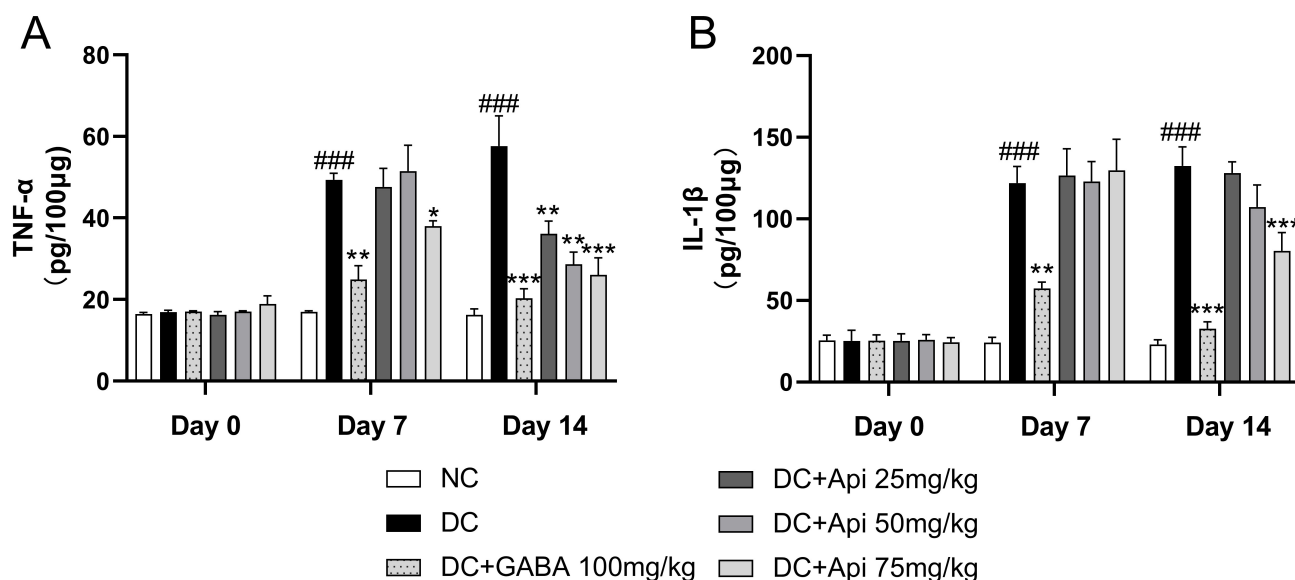


Fig. 2. Anti-inflammatory effect of Apigenin treatment was evaluated through the measurements of TNF- α (A) and IL-1 β (B) in the spinal cord of treated mice. N = 6 in all groups. Data are represented as mean $\pm$ SD. ### p < 0.001, when significantly different in comparison to NC; * p < 0.05, ** p < 0.01, *** p < 0.001, when significantly different in comparison to DC. Api, Apigenin; NC, normal control; DC, disease control; GABA, Gabapentin.

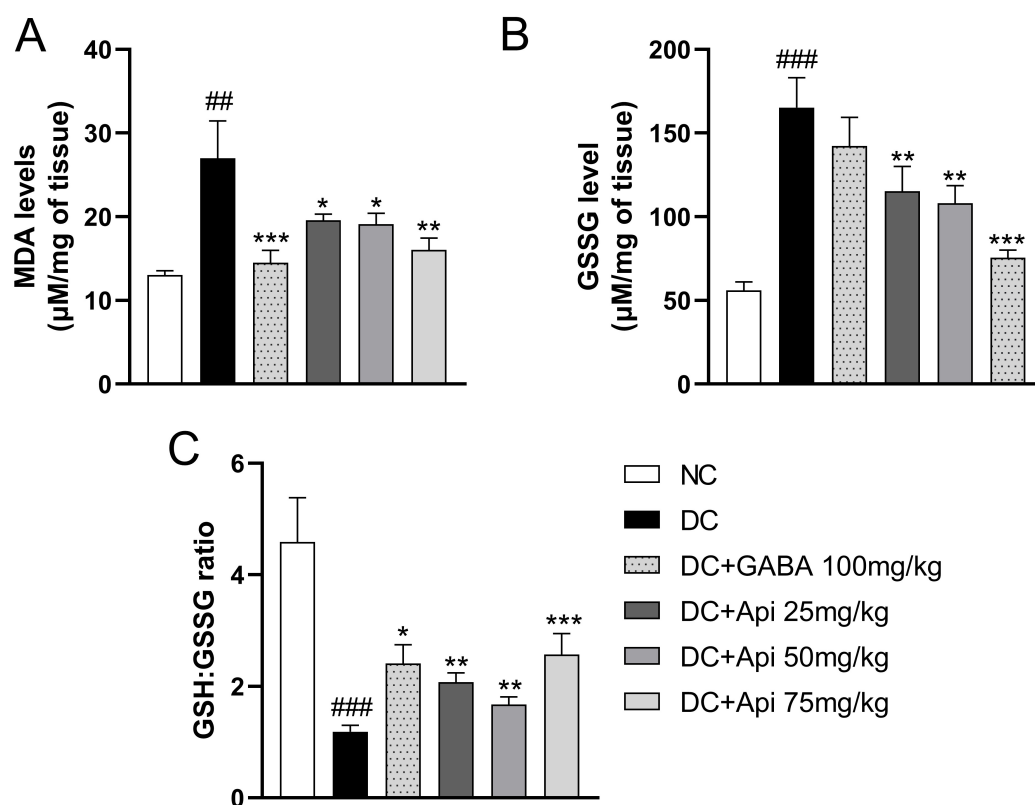


Fig. 3. Apigenin alleviated CIPN-induced oxidative stress and inflammation of CIPN mice. Effects of Apigenin treatments on the oxidative stress status in surgical spinal cord tissue were evaluated through MDA level (A), GSSG level (B), and GSH:GSSG ratio (C). Data are represented as mean $\pm$ SD. N = 6 in all groups. ## p < 0.01, ### p < 0.001, when significantly different in comparison to NC; * p < 0.05, ** p < 0.01, *** p < 0.001, when significantly different in comparison to DC. Api, Apigenin; NC, normal control; DC, disease control; GABA, Gabapentin.

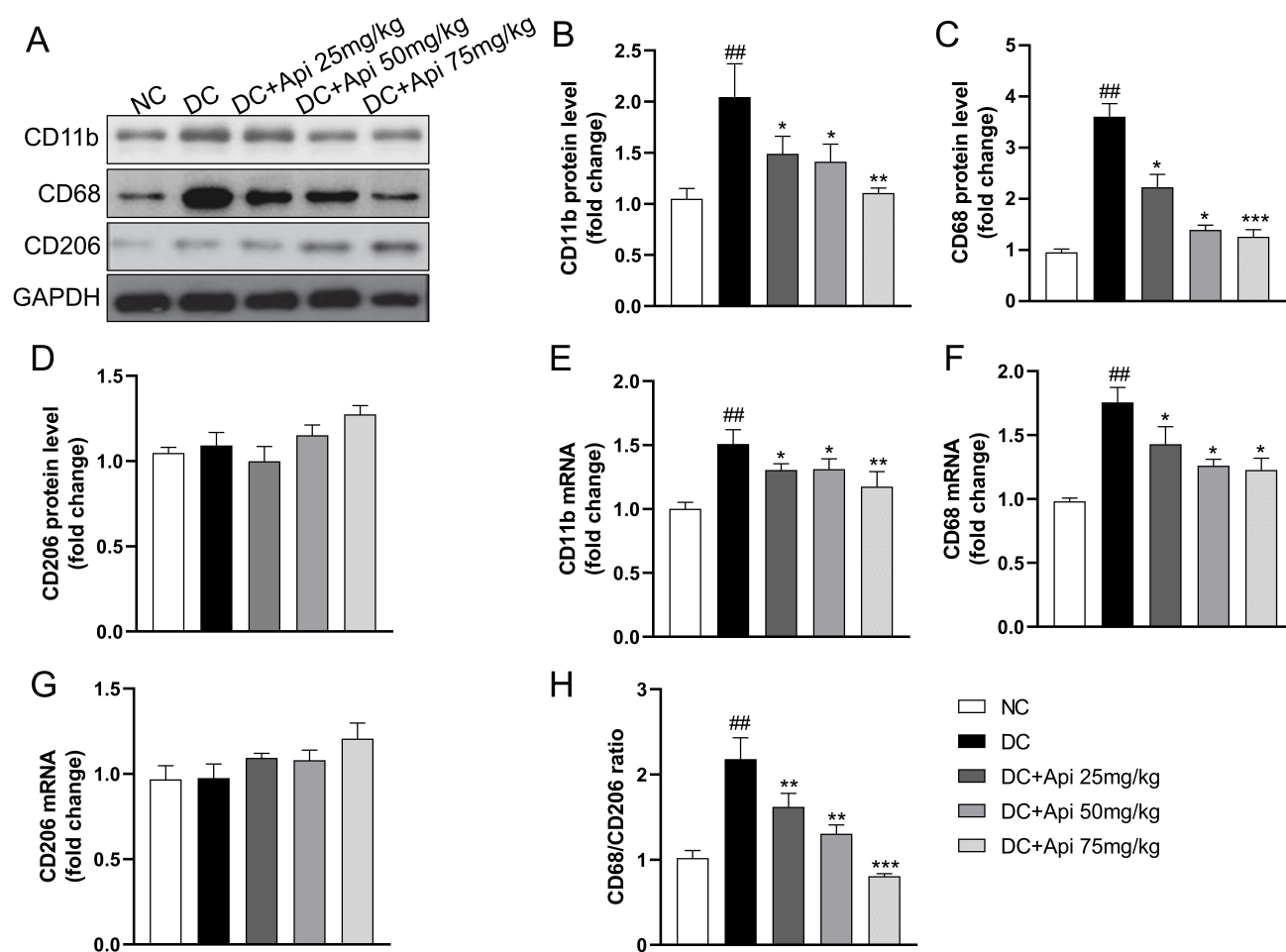


Fig. 4. Apigenin altered pro-inflammatory and anti-inflammatory phenotypes microglia markers of the nerve tissue of CIPN mice. (A) A representative blot of CD11b, CD68, CD206 of the nerve tissue. (B–D) A quantitative analysis of CD11b, CD68, CD206 protein level. Fold increase of pro-inflammatory phenotype markers (*CD11b*, *CD68*) (E,F) and anti-inflammatory phenotype microglia markers (*CD206*) (G) mRNA. (H) Fold increase of the ratio of CD68/CD206. Data are represented as mean $\pm$ SD. N = 6 in all groups. $##p < 0.01$, when significantly different in comparison to NC; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, when significantly different in comparison to DC. Api, Apigenin; NC, normal control; DC, disease control.

noted the expression of CD11b, CD68 protein, but had no significant effect on CD206, Apigenin administration significantly decreased the expression of CD68 and CD11b (Fig. 4A–D), which was further verified at the gene expression levels (Fig. 4E–G). Notably, the CD68/CD206 ratio was elevated in the CIPN group and significantly decreased after Apigenin administration (Fig. 4H). These data suggested that Apigenin suppressed pro-inflammatory and anti-inflammatory phenotypes of microglia markers in the surgical spinal cord tissue of CIPN mice.

3.5 Apigenin Increased the Nuclear Recruitment of NRF2 and Activated NRF2/ARE Signaling Pathway

Recent studies have shown that Apigenin affected the occurrence and development of various diseases through the NRF2/ARE signaling pathway [26]. We determined NRF2 protein levels in the cytoplasmic and nuclear fractions of microglia from lumbar spinal cord of control

and CIPN mice. The results showed that cytoplasmic NRF2 protein in the all CIPN groups was decreased relative to the normal group, while the nuclear NRF2 protein was increased in microglia after Apigenin administration (Fig. 5A,B). To further verify the nuclear translocation of NRF2, we detected the expression of its downstream target genes Heme oxygenase 1 (*Hmox1*) and NAD (P) H quinone oxidoreductase1 (*Nqo1*) by RT-qPCR. The results showed that the expression of *Hmox1* and *Nqo1* genes was significantly upregulated in Apigenin-treated CIPN mice in a dose-dependent manner (Fig. 5C,D). All these data indicated that Apigenin induced nuclear recruitment of NRF2 and activated the NRF2/ARE pathway in microglia.

4. Discussion

Numerous studies have investigated the effects of flavonoids on various biological phenomena and mechanisms to develop new therapeutic tools [27,28]. Apigenin

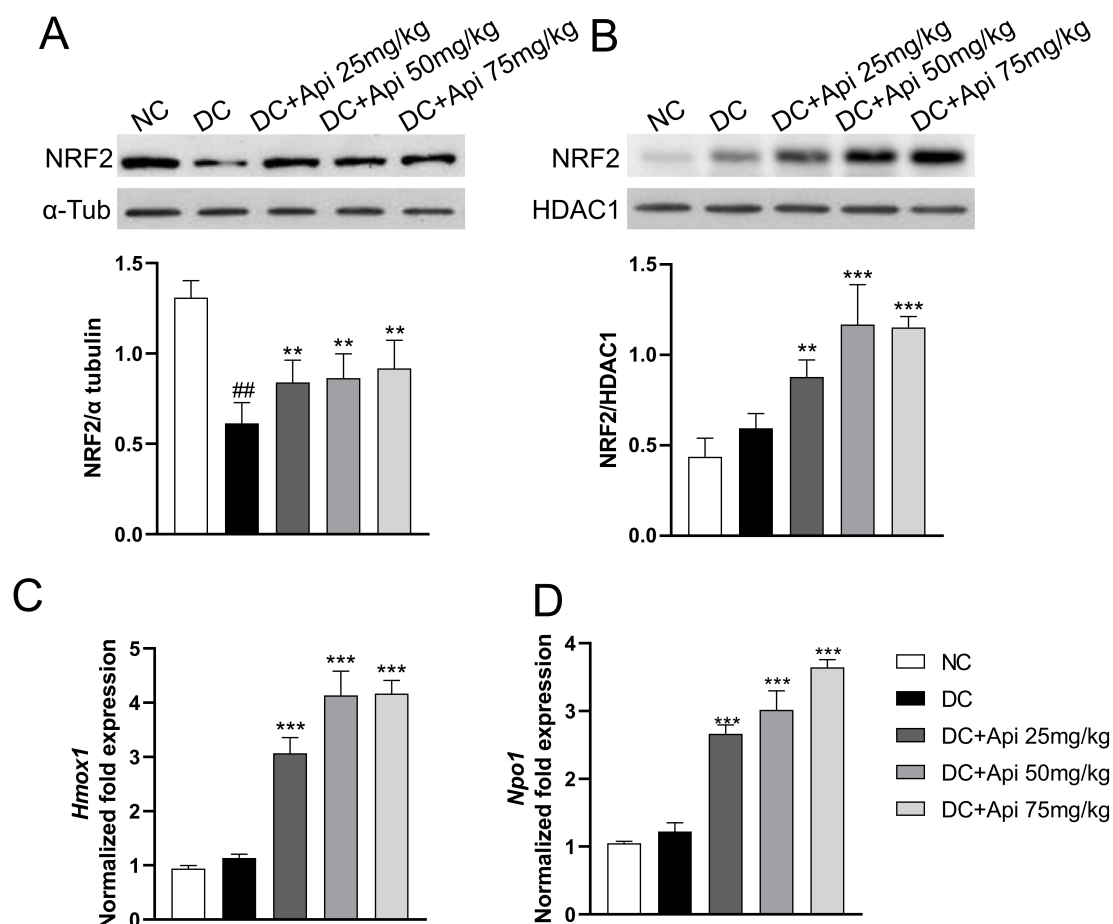


Fig. 5. Apigenin induced nuclear recruitment of NRF2. The localization of NRF2 protein was evaluated in isolated microglia from different groups. (A,B) Cytoplasmic and nuclear NRF2 protein expression (80 kDa) was evaluated in our experimental conditions. α -Tubulin (α Tub, 50 kDa) and histone deacetylase 1 (HDAC1) 55 kDa were used as reference proteins. Detected proteins were quantified and normalized. (C,D) Expression of NRF2-target genes (*Hmox1*, *Nqo1*) were evaluated by real-time PCR. Gene expressions were normalized to *Rpl27* expression level and control levels were fixed at 1.0. Data are represented as mean $\pm$ SD. ^{##} $p < 0.01$, when significantly different in comparison to NC; ^{**} $p < 0.01$, ^{***} $p < 0.001$, when significantly different in comparison to DC. Api, Apigenin; NC, normal control; DC, disease control.

has been used as an herbal medicine in China for its anti-inflammatory and anti-oxidative activities. In this study, we established the PTX-induced CIPN mouse model, investigated the protective role of Apigenin on microglia, and revealed that Apigenin relieves neuropathic pain by regulating the function of microglia.

Microglia are important neural-specific immune cells that influence brain development, neural environment maintenance, and neural repair [29]. Microglia has been reported to involve in neuropathic pain in nerve injury [30]. In neuropathic pain, neurons interact with microglia via purinergic signaling, the interference between astrocytes and microglia also causes neuropathic pain. The elevation of both oxidative stress level and inflammatory cytokine production contribute to CIPN significantly [11,12]. In line with the above findings, we found that TNF- α and IL-1 β levels in the hippocampus and spinal cord were increased markedly with PTX treatment. Meanwhile, the pro-

duction of MDA and GSSG was increased, and the ratio of GSH:GSSG in the treated mice was significantly elevated too. Strikingly, upon Apigenin treatment, both oxidative stress level and inflammatory cytokine production were decreased in surgical spinal cord tissues, which exhibited a dose-dependent pattern. Moreover, Apigenin administration significantly reduced the expression of pro-inflammatory phenotype markers (CD11b, CD68) and the ratio of CD68/CD206, which were induced after PTX treatment. All the findings indicated that Apigenin alters pro-inflammatory and anti-inflammatory phenotypes ratio of microglia in CIPN mouse.

Recent studies have shown that plant-derived active molecules such as Apigenin affect the occurrence and development of various diseases through the NRF2/ARE signaling pathway [26]. Western blot data showed that Apigenin administration significantly increased the nuclear NRF2 recruitment in microglia, which was further con-

firmed by the up-regulation of the downstream targets of NRF2/ARE signaling pathway. Given NRF2 plays an important role in regulation of the expression of antioxidant genes that exert anti-inflammatory functions [31]. We speculate that the anti-inflammation and -oxidative stress functions of Apigenin in CIPN mice are mediated by the induction of nuclear recruitment of NRF2 and activation of NRF2/ARE signaling pathway. Meanwhile, several important questions need to be addressed in following studies, such as whether the inhibitory effect of Apigenin is specifically on CIPN pain instead of all pain sensation. Also, further tail flick and hot plate tests need to be performed to establish that PTX does in fact observably lower pain threshold. The antioxidant role of Apigenin at gene expression and enzyme activity levels that related to oxidative stress regulation process could be profiled. Last, the underlying mechanism of Apigenin-induced nuclear localization of NRF2 needs more delicate experiments to elucidate.

5. Conclusions

Apigenin alleviates PTX-induced CIPN in mouse model through the regulation of inflammation and oxidative stress in microglia. This action is mediated by the Apigenin-induced nuclear localization of NRF2 and activation of NRF2/ARE pathway.

Availability of Data and Materials

Data and materials could be obtained upon reasonable request to the corresponding author.

Author Contributions

WJX, WQX, CCJ, ZMK, NZL conducted the experiments, analyzed the data, and wrote the manuscript; WJX conceived the study. All authors discussed the results and approved the final manuscript.

Ethics Approval and Consent to Participate

The animal study and treatment protocols were approved by Quanzhou First Hospital Affiliated to Fujian Medical University (365p2.v1).

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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