Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Morin Regulates M1/M2 Microglial Polarization via NF-κB p65 to Alleviate Vincristine-Induced Neuropathic Pain

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Background: Morin can alleviate vincristine-induced neuropathic pain via inhibiting neuroinflammation. Microglial cells play an important role in initiating and maintenance of pain and neuroinflammation. It remains unclear whether morin exerts antinociceptive properties through the regulation of microglial cells. This study aimed to elucidate the mechanisms of morin against neuropathic pain focusing on microglial cells.

Methods: The thermal withdrawal latency and mechanical withdrawal threshold were used as measures of pain behaviours. Histological abnormalities of the sciatic nerve were observed with transmission electron microscopy. The sciatic functional index and the sciatic nerve conduction velocity were used as measures of the functional deficits of the sciatic nerve. Inflammatory factors were detected using ELISA. The expression of M1/M2 polarization markers of microglia and nuclear factor κB (NF-κB) p65 were measured by immunofluorescence, real-time quantitative PCR and Western blotting.

Results: Morin alleviated vincristine-induced abnormal pain, sciatic nerve injury, and neuroinflammatory response in rats. Furthermore, morin decreased the expression of NF-κB P65 and M1 activation markers, increased the expression of M2 activation markers. Additionally, phorbol 12-myristate 13-acetate reversed the effects of morin on microglial polarization, the production of inflammatory factors and neuropathic pain, while ammonium pyrrolidine dithiocarbamate showed the opposite effects.

Conclusion: Our results demonstrate that morin inhibits neuroinflammation to alleviate vincristine-induced neuropathic pain via inhibiting the NF-κB signalling pathway to regulate M1/M2 microglial polarization.

Keywords: neuropathic pain, morin, microglia, NF-κB, neuroinflammation

Introduction

Neuropathic pain is chronic pain caused by lesions or diseases of the somatosensory nervous system, and neuropathic pain usually manifests as persistent spontaneous pain, hyperalgesia, ectopic pain, and sensory disturbance, severely affecting the quality of life.[1,2] At present, the pathogenesis of neuropathic pain remains unclear. It has been demonstrated that neuroinflammation is extremely important to the process of the occurrence and development of neuropathic pain.[3–5] Neuroinflammation can activate glial cells to increase the production of inflammatory factors such as interleukin (IL)-6, IL-1β and tumor necrosis factor α (TNF-α), further inducing neuroinflammation and aggravating neuropathic pain.[6,7]

Most of the current treatments for neuropathic pain rely on the traditional pharmacotherapy, such as Gabapentin, Pregabalin. However, neuropathic pain is still difficult to treat, which demands for the development of novel pharmaceutical agents. Morin is a natural bioflavonoid extracted from the *Musaceae plant* that exhibits many biological activities, including anti-inflammatory and antioxidant effects.[8–10] Studies have shown that morin effectively inhibits the inflammatory response by inhibiting nuclear factor κB (NF-κB), inhibiting the release of pro-inflammatory mediators

Received: 15 January 2024
Accepted: 14 July 2024
Published: 22 July 2024

such as IL-1β, IL-6, TNF-α.[11] Additionally, our previous study has demonstrated that morin could reduce inflammation and relieve neuropathic pain induced by vincristine.[12] However, the detailed mechanisms by which morin regulates neuroinflammation and neuropathic pain are not well elucidated. Previous studies have mainly focused on peripheral and spinal mechanisms regarding the antinociceptive properties of morin,[13] with few studies on the cortex. It was revealed that pain processing is related to cortical networks, and cerebral cortex plays an important role in pain.[14] That's why we focused cerebral cortex in this study.

Microglia are specialized macrophage cells responsible for homeostatic control of inflammation and regulating innate immunity in the central nervous system.[15,16] Microglia are prone to activation and polarization in pathological conditions.[17] Microglial polarization is closely associated with the occurrence and development of neuroinflammation. The polarization of microglia can be separated into two types based on their secreted cytokines: the proinflammatory M1 type and the anti-inflammatory M2 type.[18] The classically activated M1 phenotype can release the inflammatory factors, such as IL-6, IL-1β, TNF-α and oxidative metabolites, inducing neuroinflammation.[19] In contrast, the activated M2 phenotype can secrete a variety of protective neurotrophic factors and anti-inflammatory factors, including BDNF, TGF-β and IL-10, which can reduce local neuroinflammation and promote brain injury repair by clearing cell debris.[15,20] Specifically, microglial cells were reported to play important roles in initiating and maintenance of pain. In addition, the interactions between microglial cells and neurons have been proven extremely crucial for neuropathic pain.[21] However, it remains unclear whether the regulation of M1/M2 microglial polarization is related to the effects of morin on inhibiting neuroinflammation to alleviate vincristine-induced neuropathic pain. The current study focused on the role of microglia in the antinociceptive properties of morin, and the findings could provide interesting and alternative mechanisms for morin in pain.

As an important transcriptional regulator in cells, NF-κB is usually in the form of p50-p65 heterodimers combined with its inhibitor kappaB (IκB) and in a nonactivated state.[22] NF-κB can be activated by various stimulating elements (B-cell activating factor, lymphotoxin, and bacterial lipopolysaccharides), which can induce the expression of multiple genes and produce a variety of cytokines involved in the inflammatory response.[23] NF-κB is closely related to the occurrence of inflammatory response.[24] But more research is needed to determine whether NF-κB it is related to the regulation of microglial M1/M2 polarization by morin to alleviate neuropathic pain.

In the study, we aimed to explore the mechanisms underlying the effects of morin against vincristine-induced neuropathic pain with the focus on microglial M1/M2 polarization.

Materials and Methods

Animals

Forty-eight male Sprague-Dawley rats of SPF-grade, weighing between 350 and 400 g, were selected from the Animal Centre of Guizhou Medical University (license number SYXK (Guizhou) 2018–0001). The rats were housed in a temperature-controlled room and maintained on a 12-hour light/dark cycle. Regular monitoring was conducted to ensure the welfare of each animal. Our research protocol received approval from the Institutional Animal Care and Use Committee of the Affiliated Hospital of Guizhou Medical University. This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (8th edition, NIH).

Reagents

Vincristine (B20160, Yuanye Biotechnology, Shanghai, China) was intraperitoneally injected at 0.1 mg/mL. Morinhydrate (Y41134, Yuanye Biotechnology) was first dissolved in dimethyl sulfoxide (DMSO) and then diluted with normal saline to a concentration of 50 mg/mL for further use (final concentration of DMSO diluted was less than 5%). Phorbol 12-myristate 13-acetate (PMA; HY-18739, Med Chem Express, Monmouth Junction, NJ) and ammonium pyrrolidine dithiocarbamate (PDTC; P8765, Sigma, St. Louis, MO) were used.

Experimental Design and Drug Treatment

According to a previous study, the neuropathic pain model was constructed by injecting 0.1 mg/kg of vincristine intraperitoneally on days 1–5 and 8–12.[25] We used the hot plate test and von Frey test to observe the behavioural

changes in rats and confirm the successful establishment of the model. The administration time of the NF-κB agonist and inhibitor was consistent with that of morin at a 100 mg/kg intragastric interval of one day (days 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20). The optimal dosage of morin was based on our previous study.[12] On the 21st day after the behavioural test, rats were given 40 mg/kg 1% pentobarbital anaesthesia, the sciatic nerve was taken for nerve conduction velocity (NCV) determination, and the brain was taken for pathological and biochemical detection.

Experiment 1

We randomly divided 18 rats into three groups (n=6): the control group (Con, injection of 5% DMSO), vincristine group (Vin, vincristine at a dose of 0.1mg/kg was administered by injection), and vincristine+morin group (Vin+Morin, vincristine at a dose of 0.1 mg/kg was administered intravenously, and morin at a dose of 100 mg/kg was given orally). Behavioural tests were administrated on days 0, 3, 7, 10, 14, and 21, and brain tissues were taken from rats for biochemical tests on day 21.

Experiment 2

We randomly divided 30 rats into five groups (n=6): Control rats were injected with 5% DMSO. The rats in vincristine group were administered with 0.1 mg/kg vincristine. The rats in vincristine+morin group were given 0.1 mg/kg vincristine and intragastric administered 100 mg/kg morin. The rats in vincristine+morin+PMA group were administered with 0.1 mg/kg vincristine and 5 μg/kg PMA,[7,26] and intragastric administered 100 mg/kg morin. The rats in vincristine +morin+PDTC group were administered with 0.1 mg/kg vincristine and 50 mg/kg PDTC,[8,25] and intragastric administered 100 mg/kg morin. Behavioural tests were conducted on days 0, 3, 7, 10, 14, and 21, and parietal cerebral cortex was taken from rats for biochemical tests on day 21.

Pain Behaviour Tests

Prior to vincristine administration, baseline behavioural tests were conducted. The thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT) were assessed on days 3, 7, 10, 14, and 21 post administration.

For the Von Frey test, rats were acclimated in a transparent plastic cage with metal mesh. A Von Frey electronic pain meter was used to vertically stimulate the hind foot of the rats. The highest value recorded during paw retraction or licking behaviour was considered as the pain threshold. This stimulation and measurement process five times at intervals of 30seconds, with an average value calculated as the mechanical pain threshold.

To evaluate thermal hyperalgesia, a hot plate test was performed at a temperature maintained around approximately 52±1°C. After each rat was placed in an open round clear plastic cylinder for 30 min of acclimation, the time from placement to rear paw lifting, jumping, licking the back paw, or attempting to escape the hot plate was documented. The termination time was set to 30s to eschew tissue damage, and if the rat did not respond within 30s, the rat was removed from the cylinder, and the heat pain threshold was recorded as 30s. The test was performed every 5 min for three consecutive measurements, and the mean value was calculated.

NCV Measurement

After administering anaesthesia, the rats were positioned supine, and a longitudinal incision was made on approximately one-third of the hind leg skin bilaterally. Subsequently, gentle dissection of muscle tissue allowed for exposure of the sciatic nerve, from which we obtained the longest possible segment. Electrophysiological assessments were performed to measure NCV followed a published protocol[9,27] with slight modification. The left sciatic nerve was quickly cut open, and placed in an insulated box. Then, it was treated with a single pulse at the distal end at 200 mV intensity for 100 ms. There were two recording electrodes placed at the near-end and far-end of the sciatic nerve. The distance between these two recording electrodes was measured. The following formula was used: NCV (m/s^{-1}) = The distance between two recording electrodes/nerve conduction time between these two recording electrodes.

Transmission Electron Microscopy

On the 21st day, rats in each group underwent fixation of their sciatic nerves using a solution containing 3% glutaraldehyde, followed by additional fixation with a solution containing 1% osmium tetroxide. The samples were gradually dehydrated using acetone (30%, 50%, 70%, 80%, 90%, and finally 100%). Subsequently, thin sections measuring approximately between 60 to 90 nm were sliced from the embedded specimens using an ultrUC7rt (LEICA). To visualize the tissue sections, staining was performed with uranium acetate and lead citrate prior to examination under a transmission electron microscope (JEM-1400Flash, JEOL).

Immunofluorescence

After conducting behaviour tests on day 21, the rats were anesthetized with 1% pentobarbitone and their left ventricles were perfused with normal saline followed by paraformaldehyde fixation. Subsequently, the parietal cerebral cortex was fixed in paraformaldehyde before being immersed in sucrose solutions of varying concentrations and embedded with OCT. Finally, frozen sections measuring 12 μm thickness were obtained from the brain tissue. These sections underwent incubation with primary antibodies including IBA1 (ab178846, Abcam, Cambridge, MA), CD32 (16,559-1-AP, Proteintech, Rosemont, IL), ARG1 (DF6657, Affinity, Shanghai, China), and NF-κB P65 (AF6217, Affinity) after treatment with goat serum at room temperature for 1 hour. Following this step, the samples were washed three times using phosphate-buffered saline (PBS) prior to undergoing secondary antibody incubation at room temperature for one hour. Then the sections were subjected to three rounds of PBS washing. To visualize the cell nucleus, we employed a DAPI-containing anti-fluorescence attenuating tablet and examined the immune response using a fluorescence microscope manufactured by Carl Zeiss.

Elisa

The parietal cerebral cortex was homogenized and then centrifuged at 5000 *g* for 10 minutes to separate the supernatant from the sediment. The levels of proinflammatory factors were quantified using specific ELISA kits, namely IL-1β (RX302869R, Rui Xin Biotech, Guangzhou, China) and IL-6 (RX302856R, Rui Xin Biotech), while the anti-inflammatory factors IL-4 (RX302858R, Rui Xin Biotech) and IL-10 (RX302880R, Rui Xin Biotech) were measured.

Real-Time Quantitative PCR

According to the provided instructions, RNA extraction from parietal cerebral cortex was performed using TriQuick Reagent (R1100, Solarbio, Beijing, China). Reverse transcription of RNA was carried out using PrimeScriptTM RT Reagent Kit with gDNA Eraser (RR047A, Takara, Dalian, China) to generate cDNA. Subsequently, RNA amplification was conducted using TBGreen Premix ExTaqTM II. The primer sequences (synthesized by Shanghai Sheng Gong Biological Co., Ltd, Shanghai, China) utilized in this study are presented as followed (5'-3'):

GAPDH:
Forward: CAAGTTCAACGGCACAGTCAAGG;
Reverse: ACATACTCAGCACCAGCATCACC.
CD32:
Forward: AAGGAAGACACTGTGACACTGATG;
Reverse: TGTAGTTGGCTTGGGCTTGATG.
ARG1:
Forward: GTGTGGTGCTGGGTGGAGAC;
Reverse: GCGGAGTGTTGATGTCAGTGTG.
NF-κB P65:
Forward: GGATGGCTTCTATGAGGCTGAAC;
Reverse: TGCTCCAGGTCTCGCTTCTTC

Data analysis involved employing the $2^{-\Delta\Delta CT}$ method.

Western Blot

The BCA protein quantification was performed following complete lysis of parietal cerebral cortex using a lysate containing protease inhibitor. Subsequently, the proteins were separated by electrophoresis on a 10% SDS gel under constant pressures of 80 V for the concentrating gel and 120 V for the separation gel. Afterwards, the proteins were transferred onto polyvinylidene fluoride membranes. The samples were then blocked with 5% skim milk for one hour and incubated overnight at 4°C with NF-κB P65 (1:1000, AF2006, Affinity), CD32 (1:500, 15,625-1-AP), and CD32 (1:500, 15,625-1-AP). Additionally, DF6657 antibody (Affinity) and GAPDH antibody (1:10,000, 10,494-1-AP, Proteintech) were used. These blots were subsequently probed with horseradish peroxidase-coupled secondary antibody (1:5000 SA00001-2 Proteintech) at room temperature for one hour before being automatically developed in a gel imaging system utilizing an ECL luminescence kit (MA0186 Meilunbio). ImageJ software from the National Institutes of Health in the USA was employed to analyze the gray values of Western blot bands.

Statistical Analysis

GraphPad Prism statistical software (version 9.3, GraphPad Software) was employed for data analysis. The sample size for the animal experiments was calculated based on the Resource Equation Approach.[28] According to the calculation formula, the recommended sample size for animal experiments was 5 to 7 animals per group. In consideration of the requirement for diverse biochemical assays, we used a sample size of n=6 per group, which aligns with the computational algorithm. Q-Q plots were utilized to evaluate data distribution prior to analyses (results were shown in the Supplementary Materials). The results were presented as mean ± standard deviation (SD). Differences among three or more groups were examined by one-way analysis of variance (ANOVA) or Welch ANOVA. Fisher's least significant difference (LSD) test for ANOVA or Games-Howell test for Welch ANOVA was performed for multiple comparisons between groups. The experiments were independently repeated in triplicate to confirm the findings. A significance threshold of $p < 0.05$ was applied.

Results

Morin Alleviates Vincristine-Induced Allodynia

The experimental procedures were detailed in Figure 1A. The MWT and the TWL of the rats were assessed using a Von Frey electronic pain meter and hot plate meter, respectively, as illustrated in Figure 1B and C. Prior to treatment, no significant differences observed in the MWT and TWL among all groups ($p > 0.05$). Following a three-day intraperitoneal injection of vincristine, the rats exhibited significantly reduced MWT and TWL compared to the control group ($p < 0.05$) (Control group at day 21: MWT: 59.21±1.71 g; TWL: 10.75±0.74 s. Vincristine group at day 21: MWT: 40.94 ±2.65 g; TWL: 4.81±0.58 s), indicating successful establishment of a neuropathic pain model by vincristine administration. Importantly, intervention with morin at a dosage of 100 mg/kg effectively alleviated abnormal mechanical pain induced by vincristine when compared to the model group ($p < 0.05$) (at day 21: MWT: 49.09±1.39 g; TWL: 8.03±0.42 s). Overall, morin demonstrated its potential in mitigating vincristine-induced abnormal pain.

Morin Alleviates the Sciatic Nerve Injury Induced by Vincristine

On the 21st day, the sciatic nerve was taken for NCV measurement. Compared with the control group (63.85±1.35 m/s), the sciatic NCV of rats was slowed in the vincristine group (42.20±1.49 m/s) ($p < 0.05$), indicating that nerve injury had been induced by vincristine. Compared with the vincristine group, the reduction in sciatic NCV induced by vincristine was significantly alleviated after 100 mg/kg morin administration (52.83±1.69 m/s) ($p < 0.05$), indicating that nerve injury was alleviated after morin treatment (Figure 2A).

We used transmission electron microscopy to observe the ultrastructure of the sciatic nerve in each group (Figure 2B). The myelin sheath of the nerve fibers in the control rats was dense, uniform and regular, with a concentric sheet-like structure alternating between light and dark, and the axonal structure was normal. In the vincristine group, the nerve injury was severe. Many nerve fibers showed a loose myelin lamellar structure, enlarged spaces, and disorderly arrangement; the collapse fracture of the plate structure and the distortion of the

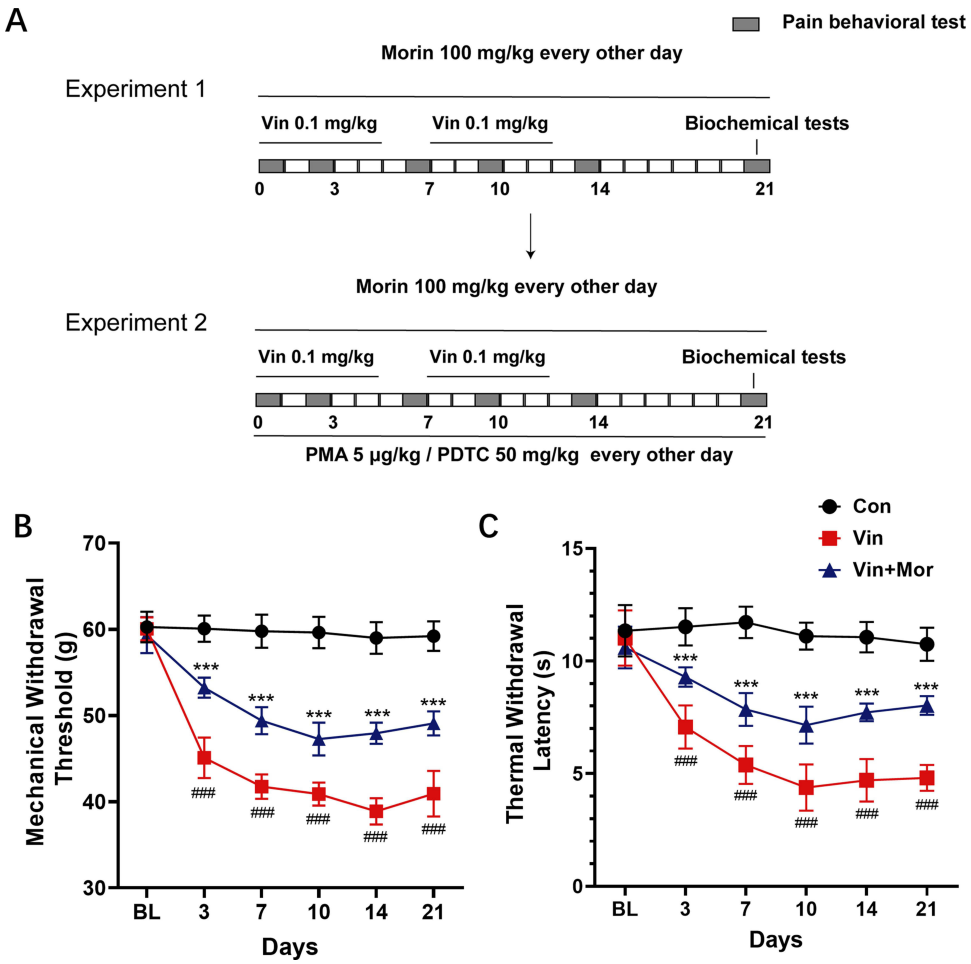

Figure 1 Morin alleviates vincristine induced allodynia. (**A**) The experimental protocol of this study. (**B** and **C**) Changes in MWT and TWL of rats in each group. n=6 rats for each group. ***$p < 0.001$ compared with control group, ###$p < 0.001$ compared with vincristine group. Data were shown as mean ± SD.

plate structure were vortex-like, and cavitation degeneration occurred. Schwann cell mitochondria were swollen and vacuolated. Compared with vincristine group, the injury of the sciatic nerve after morin treatment was significantly reduced. The structure of the nerve fiber myelin lamina was loose, the gap was enlarged, and the mitochondrial structure of Schwann cells was normal. These results demonstrate the neuroprotective function of morin.

Morin Alleviates Vincristine-Induced Neuroinflammation

To investigate the potential impact of morin on inflammatory factor levels, ELISA was employed to quantify concentrations of proinflammatory and anti-inflammatory factors in rat brain tissue. The results revealed that vincristine treatment resulted in increased expression levels of proinflammatory factors (IL-6: 197.75±13.20 pg/mL; IL-1β: 95.38±8.90 pg/mL, Figure 2C) and decreased expression levels of anti-inflammatory factors (IL-4: 48.75±3.98 pg/mL; IL-10: 180.73±12.05 pg/mL) compared to the control group (IL-6: 97.59±8.80 pg/mL; IL-1β: 44.67±3.50 pg/mL; IL-4: 105.87±9.03 pg/mL; IL-10: 422.68±29.33 pg/mL) (all $p < 0.05$). However, administration of 100 mg/kg morin (IL-6: 115.63±9.40 pg/mL; IL-1β: 62.48±4.90 pg/mL; IL-4: 80.52±6.37 pg/mL; IL-10: 318.69±26.88 pg/mL) exhibited inhibitory effects on pro-inflammatory factor production while promoting the expression of anti-inflammatory factors when compared to the vincristine group (all $p < 0.05$). However, morin treatment failed to fully restore the production of cytokines to the normal control level.

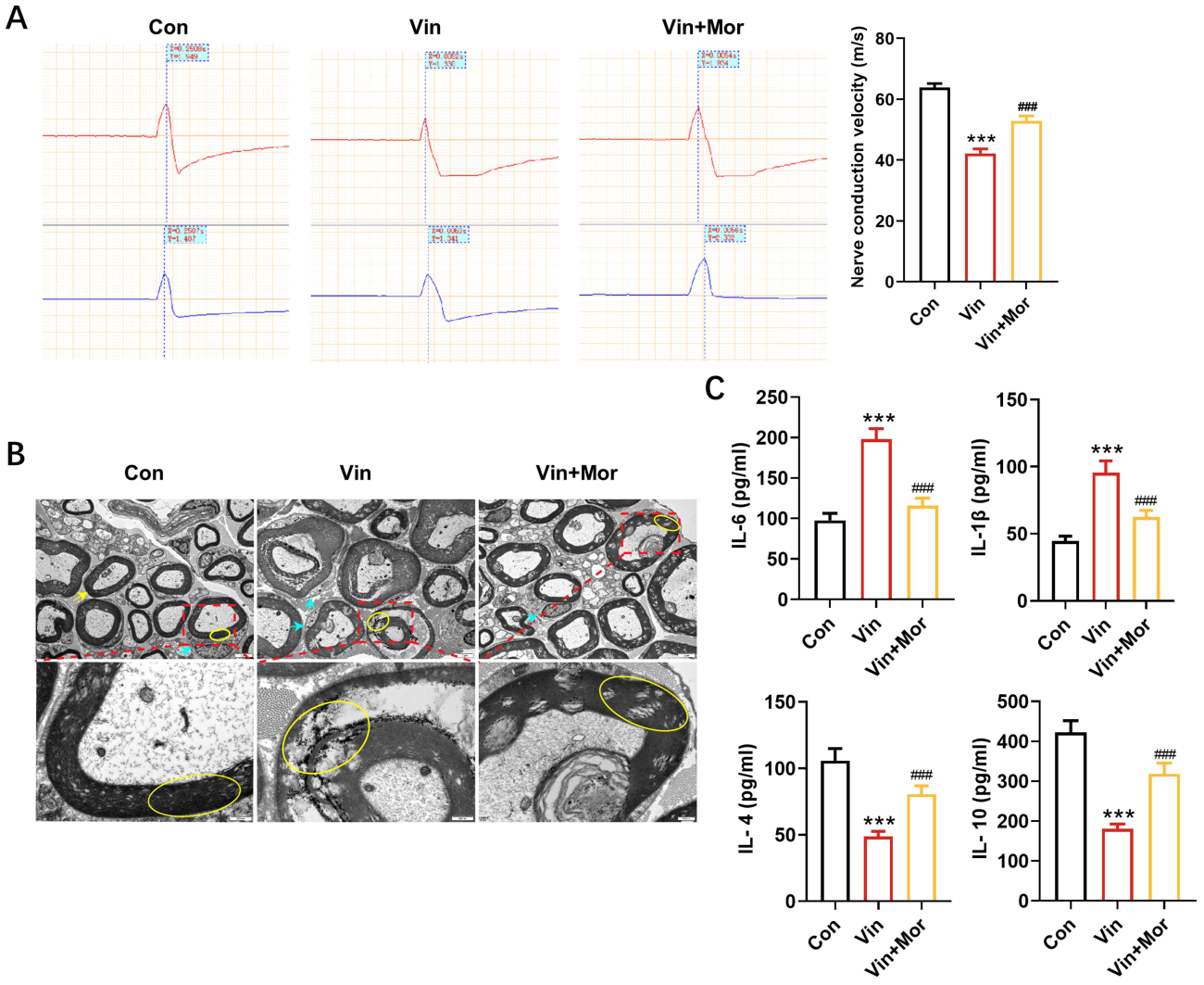

Figure 2 Morin alleviates vincristine-induced sciatic nerve injury. (**A**) Changes of sciatic nerve conduction velocity in each group. n=6 rats for each group. ***$p < 0.001$ compared with control group, ###$p < 0.001$ compared with vincristine group. Upper and down panels represented the action potentials recorded at the near-end and far-end of the sciatic nerve, respectively. (**B**) Representative images of rat sciatic nerve transmission electron microscope (yellow ↑, ○: myelin sheath, cyan ↑: Schwann cells). (**C**) Changes in the concentrations of pro-inflammatory factors and anti-inflammatory factors in cerebral cortex of rats in each group. n=6 rats for each group. ***$p < 0.001$ compared with control group, ###$p < 0.001$ compared with vincristine group. Data were shown as mean ± SD.

Effect of Morin on the M1/M2 Polarization of Microglia

To investigate the impact of morin on microglial polarization, brain tissues from rats were collected on day 21 in each group to assess the protein and mRNA levels of IBA1, a marker for activation; CD32, a marker for M1 polarization; and ARG1, a marker for M2 polarization in the cerebral cortex. In the vincristine group, downregulation in ARG1 expression, including the ARG1 fluorescence intensity (Figure 3A and B), ARG1 mRNA expression (Figure 3C) and protein expression (Figure 3D) within microglia was observed. Morin treatment (100 mg/kg) partially reversed the down-regulation of ARG1 expression. Conversely, there was an upregulation of IBA1 and CD32 expression in the vincristine group, as evidenced by their fluorescence intensity, mRNA and protein expression (Figure 3E–J). Similarly, administration of 100 mg/kg morin effectively counteracted these effects induced by vincristine. These findings underscore the ability of morin to modulate the M1/M2 microglial polarization triggered by vincristine.

Morin Affects M1/M2 Microglial Polarization by Regulating NF-κB

The NF-κB signalling pathway serves an important function in controlling the inflammatory response, and activation of this pathway is crucial to the occurrence of the neuroinflammatory response.[29] The expression of NF-κB P65 in the

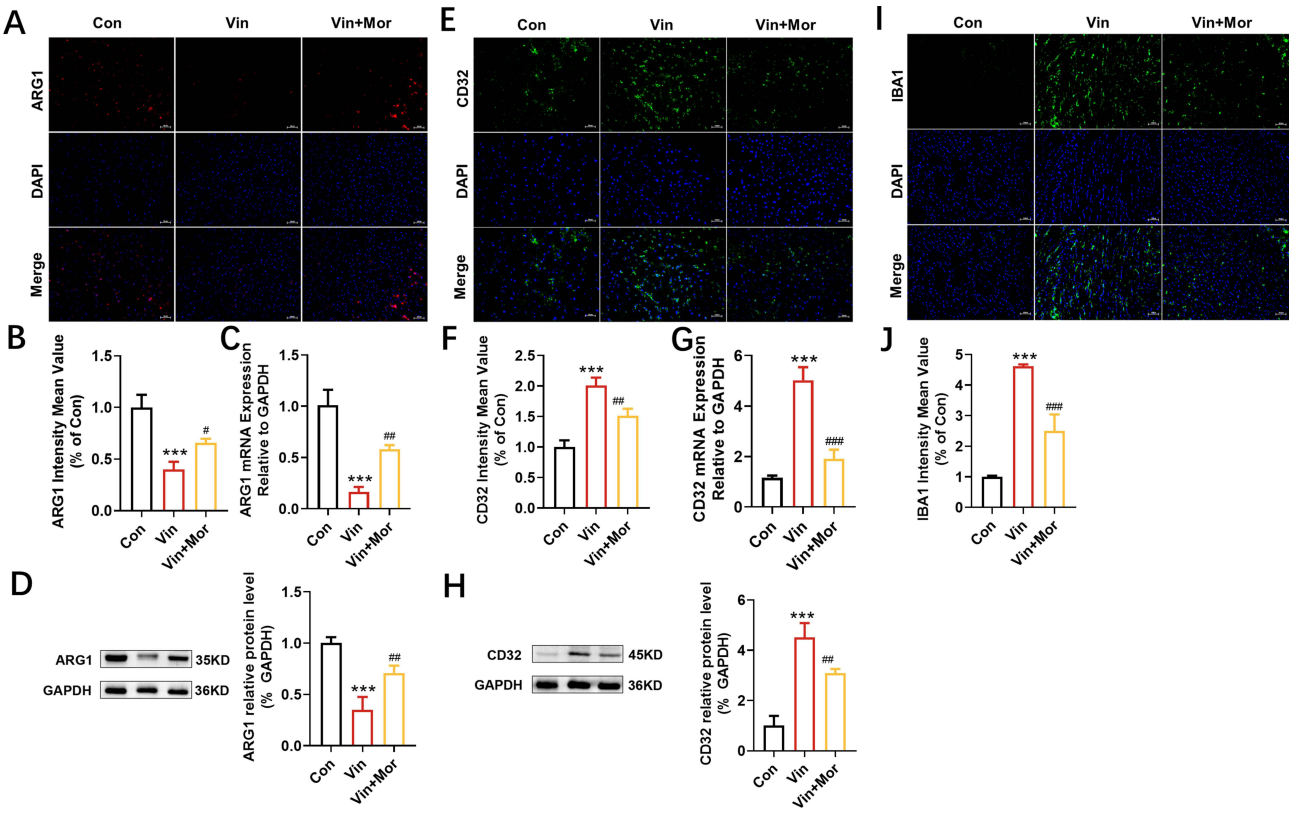

Figure 3 Morin attenuates vincristine-induced neuroinflammation by regulating M1/M2 polarization of microglia. (**A, E, I**) Representative images of ARG1, CD32 and IBA1 and (**B, F, J**) their relative fluorescence intensity in the cerebral cortex of the rats, determined by immunofluorescence staining. (**C, G**) mRNA expression of ARG1 and CD32 in the cerebral cortex of the rats, determined by real-time quantitative PCR. (**D, H**) protein expression of ARG1 and CD32 in the cerebral cortex of the rats, analyzed by Western blot. Scale = 50 μm. n=6 slices for each group for the fluorescence intensity calculation. n=6 for the PCR and Western blot assays. $^{***}p < 0.001$ compared with control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with vincristine group. Data were shown as mean ± SD.

cerebral cortex was determined by immunofluorescence and Western blotting. After 12 days of induction with vincristine, NF-κB P65 expression was significantly higher than that in the control group ($p < 0.05$), as evidenced by the NF-κB P65 fluorescence intensity (Figure 4A and B), NF-κB P65 mRNA expression (Figure 4C) and protein expression (Figure 4D). However, 100 mg/kg morin treatment reduced the expression of NF-κB P65 induced by vincristine ($p < 0.05$) (Figure 4).

To further investigate the potential correlation between microglial polarization and NF-κB, we utilized the NF-κB inhibitor PDTC and the NF-κB agonist PMA to modulate NF-κB expression. We evaluated the levels of ARG1, CD32, P65 and IBA1 in microglia. Vincristine induced a decreased expression of ARG1 in the microglia (Figure 5A–D), but an increased expression of CD32 (Figure 5E–H), NF-κB P65 (Figure 5I–L) and IBA1 expression (Figure 5M and N) in rat brain tissue, as proved by their fluorescence intensity, mRNA and protein expression, respectively. Treatment with 100 mg/kg morin partially mitigated these changes. Moreover, compared to the morin group, administration of the NF-κB agonist PMA resulted in increased expression of CD32 and IBA1, administration of the NF-κB inhibitor PDTC exhibited opposite effects. These results suggest that morin exerts inhibitory effects on M1 polarization while promoting M2 polarization of microglia through modulation of NF-κB expression.

Effects of Morin on Pain Behaviour After Regulating NF-κB Pathway

The effects of morin on neuropathic pain was explored by measuring MWT and TWL, with a focus on regulating the M1/M2 polarization of microglia via the NF-κB pathway. The results showed that vincristine significantly shortened the MWT (Control group at day 21: 61.16±1.45 g; Vincristine group at day 21: 40.40±1.49 g) and TWL (Control group at day 21: 11.01±0.61 s; Vincristine group at day 21: 4.74±0.50s) in rats, and 100 mg/kg morin intervention enhanced the MWT (Vincristine+morin group at day 21: 48.07±1.14 g) and TWL (Vincristine+morin group at day 21: 8.03±0.37 s)

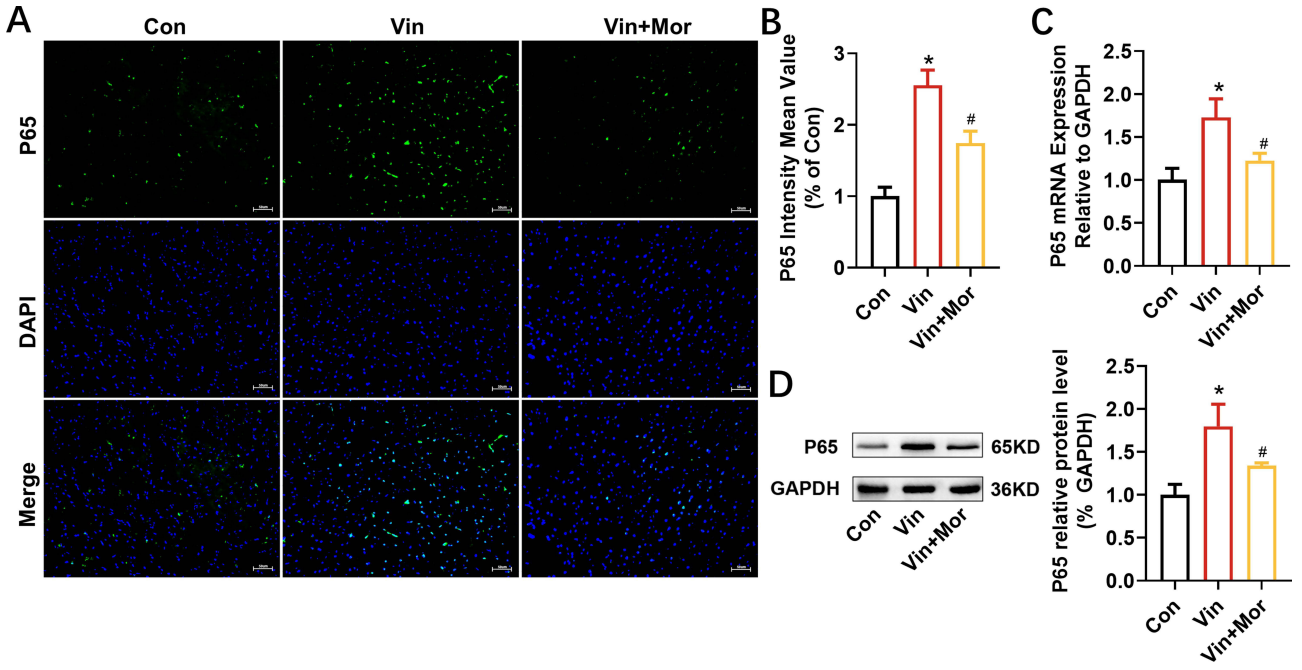

Figure 4 Effect of morin on NF-κB in neuropathic pain rat cerebral cortex. (**A**) Representative images of NF-κB P65 in cerebral cortex of rats in each group. (**B**) Relative P65 fluorescence intensity in each group. (**C**) mRNA and (**D**) protein expression of NF-κB P65 in each group. n=6 slices for each group for the fluorescence intensity calculation. n=6 for the PCR and Western blot assays. $^{*}p < 0.05$ compared with control group, $^{\#}p < 0.05$ compared with vincristine group. Data were shown as mean ± SD.

(Figure 6A and B). However, compared with the morin group, MWL and TWL were declined in the PMA group (43.66 ±1.76 g and 6.65±0.44 s for MWL and TWL at day 21, respectively), while the NF-κB inhibitor PDTC group showed the opposite effect (52.4±1.44 g and 9.29±0.47 s for MWL and TWL at day 21, respectively) (Figure 6A and B).

Effects of Morin on Neuroinflammation After the Regulation of NF-κB

To investigate the effect of morin on regulating neuroinflammation after regulating NF-κB, the concentrations of proinflammatory factors (IL-6 and IL-1β) and anti-inflammatory factors (IL-10 and IL-4) in rat brain tissue were measured by ELISA. The expression levels of IL-1β and IL-6 were increased and those of IL-4 and IL-10 were decreased in the PMA group (IL-1β: 89.23±6.78 pg/mL; IL-6: 187.20±12.50 pg/mL; IL-4: 67.97±5.71 pg/mL; IL-10: 253.50±12.30 pg/mL) when compared with the morin group (IL-1β: 72.25±6.12 pg/mL; IL-6: 158.65±8.80 pg/mL; IL-4: 87.44±6.84 pg/mL; IL-10: 328.75±22.50 pg/mL), meanwhile, the PDTC group showed the opposite effect (IL-1β: 55.03 ±4.54 pg/mL; IL-6: 124.34±9.34 pg/mL; IL-4: 107.45±8.89 pg/mL; IL-10: 397.65±30.14 pg/mL) (Figure 6C).

Morin Alleviates Vincristine-Induced Nerve Injury by Regulating NF-κB

After intervention with the NF-κB inhibitor PDTC and the agonist PMA, an animal model of neuropathic pain was established with vincristine, and 100 mg/kg morin was given by intragastric administration. The sciatic NCV of rats in each group was measured. The results indicated that compared with that of morin group (52.50±2.21 m/s), the sciatic NCV of the PMA group (41.90±1.65 m/s) was decreased significantly ($p < 0.001$), while that of PDTC group (57.94 ±0.69 m/s) was increased significantly ($p < 0.05$) (Figure 7A and B).

The ultrastructure of the sciatic nerve in each group was observed using transmission electron microscopy (Figure 7C). The myelin sheath of nerve fibers in control group was compact, uniform and regular in shape, and the axon structure was normal. The nerve injury in vincristine group was severe: many nerve fibers showed a loose myelin lamellar structure and disorderly arrangement; the slab structure was collapsed and fractured, presented a vortex shape, and exhibited cavitation degeneration. Schwann cell mitochondria were swollen and vacuolated. Compared with vincristine group, the injury of the sciatic nerve in morin group was significantly reduced after treatment: the structure of the nerve fibre myelin lamina was loose, the gap was enlarged, and the mitochondrial structure of Schwann cells was

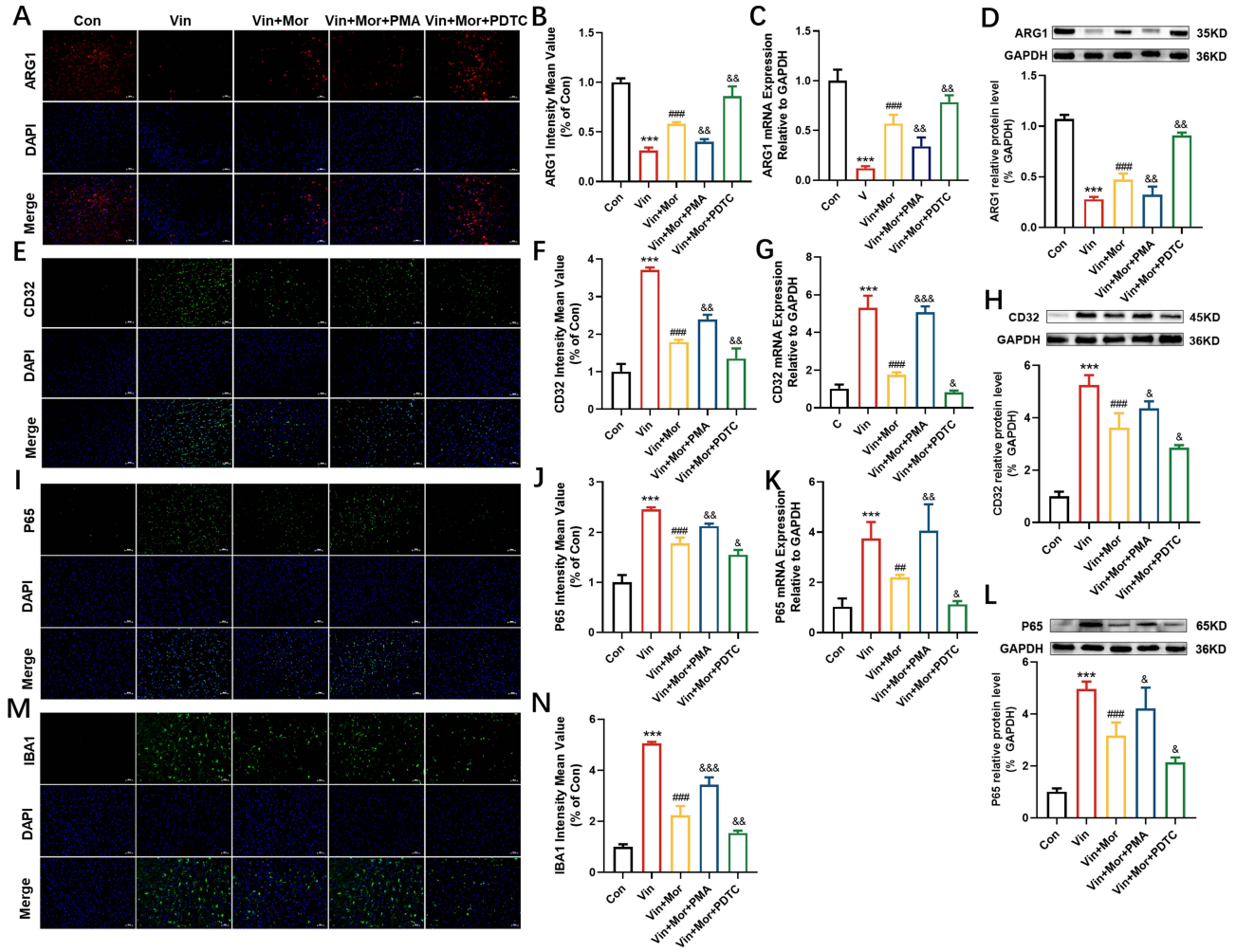

Figure 5 Morin affects microglia M1/M2 polarization by regulating NF-κB signaling. (**A, E, I, M**) Immunofluorescence staining for ARG1, CD32, P65 and IBA1 in cerebral cortex of rats in each group. Scale = 50 μm. (**B, F, J, N**) Relative ARG1, CD32, P65 and IBA1 fluorescence intensity in each group. (**C, G, K**) mRNA expression of ARG1, CD32, P65 in cerebral cortex of rats in each group, determined by real-time quantitative PCR. (**D, H, L**) Protein expression of ARG1, CD32, P65 in cerebral cortex of rats in each group, determined by Western blot. n=6 slices for each group for the fluorescence intensity calculation. n=6 for the PCR and Western blot assays. $^{***}p < 0.001$ compared to control group, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to vincristine group, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ compared to group V+M group. Data were shown as mean ± SD.

normal. In the NF-κB agonist group, the nerve damage was more serious: many nerves fibre myelin lamellar structures were loose, the gap was enlarged, the arrangement was disorderly, the lamellar structure was twisted and swirled, and cavitation degeneration appeared. The axon structure was complete. Schwann's cell structure was normal. In the NF-κB inhibitor group, the nerve damage was mild: the lamellar structure of the myelin structure of some nerve fibres was loose, there was no obvious atrophy of axons, and the structure was complete. Schwann cells showed a more normal structure.

Discussion

As an effective chemotherapeutic agent, vincristine is often used to treat many types of cancer. However, vincristine can cause dose-dependent peripheral neurotoxicity, inducing nerve damage and neuropathic pain.[1,30] In fact, one of the side effects of vincristine is peripheral neuropathy. Peripheral nerve injury often leads to central sensitization of the spinal cord, supraspinal and cortical areas of the brain, and causes central neuropathic pain through corticospinal projections. That's why we focused on cerebral cortex in the current study. In addition, vincristine intervention was reported to promote the release of proinflammatory mediators, including IL-1β, IL-6 and TNF-α, by activating microglia, astrocytes and immune cells in the dorsal horn of the spinal cord.[2,29] These inflammatory mediators could promote the

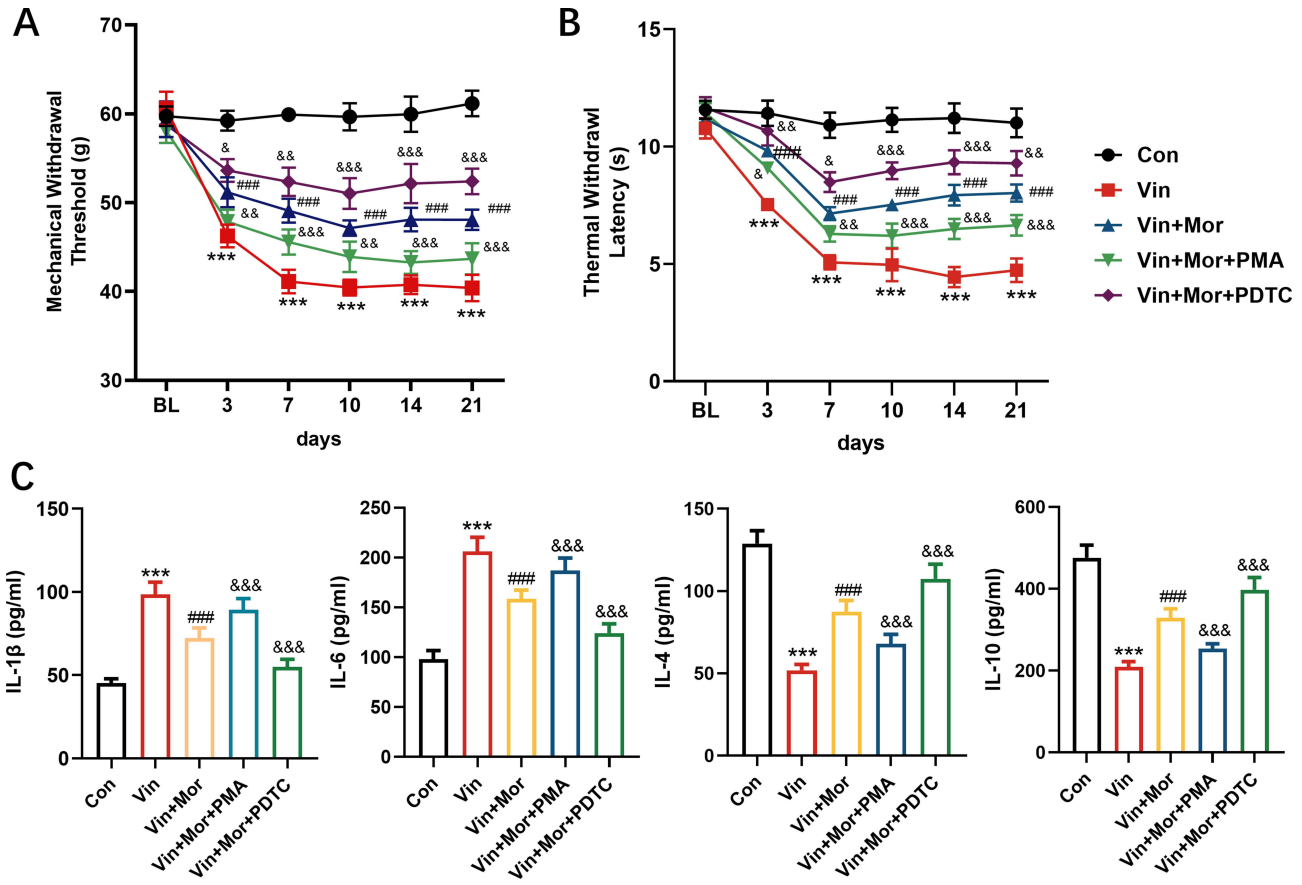

Figure 6 Effect of NF-κB regulation by morin on pain behavior. (**A** and **B**) MWT and TWL changes in each group. (**C**) The concentration changes of pro-inflammatory factors and anti-inflammatory factors in cerebral cortex of rats in each group. n=6 rats for each group. $^{***}p < 0.001$ compared with control group; $^{\#\#\#}p < 0.001$ compared with vincristine group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ compared with vincristine+morin group. Data were shown as mean ± SD.

neuroinflammatory response, which plays an important role in the occurrence and maintenance of neuropathic pain.[2,29] Inhibiting the inflammatory response is one of the clinical treatments for neuropathic pain caused by vincristine.

In vitro and in vivo studies have shown that morin has extremely low levels of toxicity and its long-term administration is well tolerated,[4,31,32] proving its potential as a promising natural drug in clinic. Numerous pieces of evidence have shown that morin may have beneficial effects on various human diseases. Specifically, Morin demonstrates antibacterial, antihypertensive, antioxidant, antitumoral, anti-inflammatory, antidiabetic, hypouricemic and neuroprotective properties,[3,31] further suggesting its use in clinical application. In the current study, the anti-neuroinflammatory activity of morin was elucidated, and the results demonstrate the potential of morin for the treatment of neuropathic pain in the clinical application.

Morin is a bioflavonoid with anti-inflammatory effects. Current findings also reveal the anti-inflammation ability of morin, as suggested by the decreased expression of pro-inflammatory factors IL-1β and IL-6, and increased expression of the anti-inflammatory factors IL-4 and IL-10. Morin seems to have no specific target and binding receptors, as demonstrated by different studies. For example, morin augmented Akt and insulin receptors phosphorylation in HepG2 cells;[5,33] It was reported to exert anti-arthritic effects via activating peroxisome proliferator-activated receptor-γ;[6,34] Specifically, morin was found to activate CB2 receptor in spinal cord to relieve bone cancer pain.[13] It was demonstrated that morin has been shown to inhibit inflammation through mitogen-activated protein kinase (MAPK) signalling pathways.[7,35]

Microglia are the first line of defense in the central nervous system and play a crucial role in the normal function of the brain and the formation of neural circuits.[9,36,37] In recent years, studies have shown that the activation of microglia is crucial to the process of neuropathic pain and has become a research hotspot in this field.[7,38,39] When a nerve is damaged, microglia are activated to the M1 phenotype, promoting the expression of inflammatory factors, resulting in neuropathic pain.[1,40] In addition, M2 polarization of microglia can alleviate neuropathic pain associated with Alzheimer's disease.[2,39]

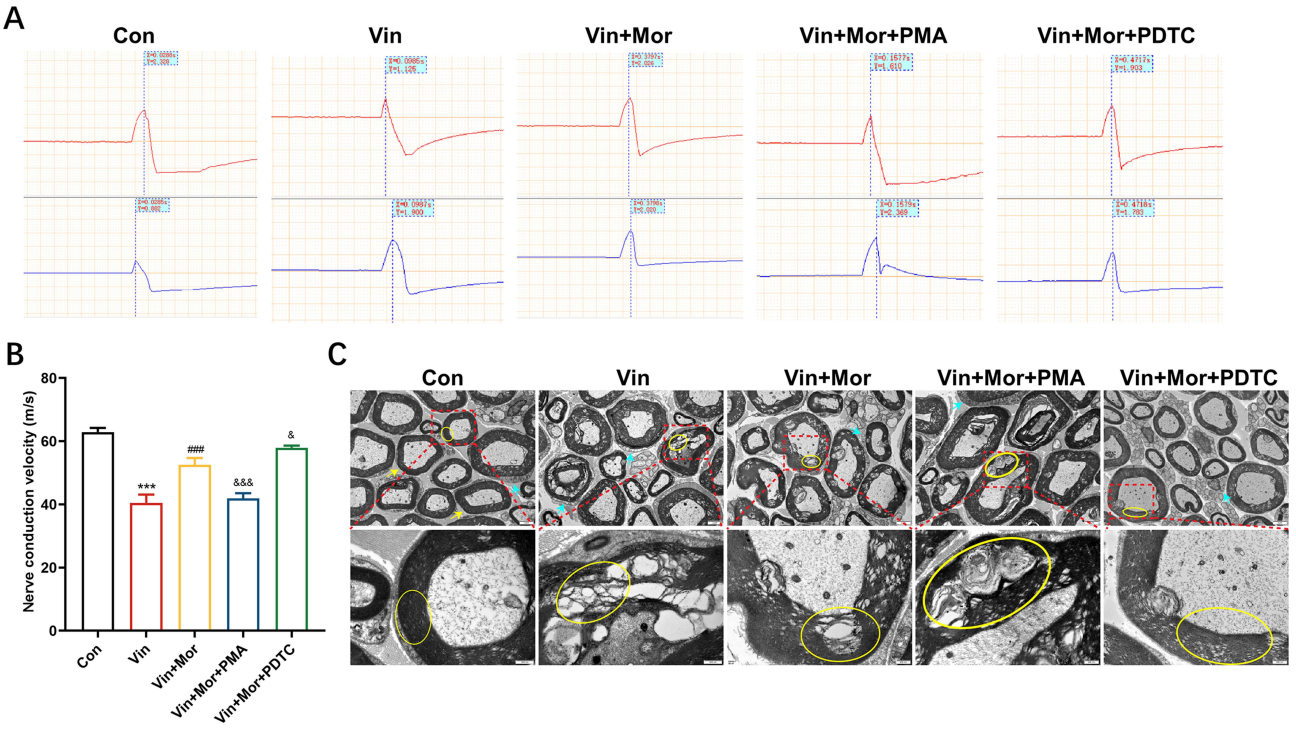

Figure 7 Morin alleviates vincristine-induced nerve injury by regulating NF-κB. (**A** and **B**) Changes in sciatic nerve conduction velocity in each group. n=6 rats for each group. Data were shown as mean ± SD. Upper and down panels represented the action potentials recorded at the near-end and far-end of the sciatic nerve, respectively. $^{***}p < 0.001$ compared with control group, $^{\#\#\#}p < 0.001$ compared with vincristine group, $^{\&}p < 0.05$ and $^{\&\&\&}p < 0.001$ compared with vincristine+morin group. (**C**) Representative images of rat sciatic nerve transmission electron microscope (yellow ↑, ○: myelin sheath, cyan ↑: Schwann cells).

Therefore, the regulation of the M1/M2 polarization of microglia may be related to the relief of neuropathic pain symptoms. Our study demonstrated that the expression levels of the microglial activation marker IBA1 and the M1 polarization marker CD32 were increased significantly in the vincristine group compared with the control group, while the expression levels of the M2 polarization marker ARG1 were decreased. Moreover, 100 mg/kg morin treatment relieved the increased levels of IBA1 and CD32 and the decreased level of ARG1 induced by vincristine. The above results showed that morin treatment could inhibit M1 microglial polarization, promote M2 polarization, reduce neuroinflammation, and relieve neuropathic pain.

NF-κB is an important signalling pathway related to inflammation. NF-κB is usually inactive in a heterodimer state, and when stimulated, the phosphorylation of IκB leads to the activation of NF-κB in nucleus to regulate the release of proinflammatory factors.[3,41] The inhibition of the NF-κB pathway can also reduce the inflammatory response and alleviate neuropathic pain.[4,42] However, it is uncertain whether NF-κB is involved in the regulation of M1/M2 microglial polarization, the inhibition of the inflammatory response, and the relief of vincristine-induced neuropathic pain. In our study, an NF-κB agonist and inhibitor were used to regulate the expression of NF-κB. These results indicated that compared with the morin intervention group, NF-κB agonist enhanced mechanical pain, increased proinflammatory factors and promoted the M1 polarization of microglia, which reversed the anti-inflammatory and neuroprotective effects of morin. NF-κB inhibitors showed the opposite effect as agonists. These results demonstrated that NF-κB is the key pathway by which morin regulates M1/M2 microglial polarization. In summary, morin can reduce the expression of NF-κB, inhibit the M1 polarization of microglia, promote M2 polarization to reduce the inflammatory response, and thus improve neuropathic pain.

There are several limitations in our study. First, we measured some, but not all, markers of M1/M2 polarization in microglia. Second, due to morin's regulatory effect on microglial polarization, we did not use minocycline to intervene in microglial polarization. Third, our study confirmed the effects of only the NF-κB pathway on the regulation of M1/M2 microglial polarization; other signalling pathways were not explored. In future studies, additional signalling pathways

need to be further investigated to identify more drug targets for the treatment of neuropathic pain. Last, based on the current findings, it is difficult to clarify whether it is a central change caused by the periphery or a peripheral change caused by the brain, which needs to be further explored with more delicate experiments.

Conclusion

In summary, our results demonstrate that morin inhibits neuroinflammation to alleviate the vincristine-induced neuropathic pain via regulating M1/M2 microglial polarization and the NF-κB signalling pathway.

Funding

This study was supported by the National Natural Science Foundation of China (Grant No. 82060220), the Outstanding reserve talents in the discipline of Affiliated Hospital of Guizhou Medical University in 2023 (gyfyxkrc-2023-15), and the science and technology plan project of Guizhou province [ZK (2023)- General 390].

Disclosure

Yi Shao and Yunfu Chen contributed equally to this paper and are co-first authors.

The authors declare that they have no conflict of interest.

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