Doi.org/10.36721/PJPS.2025.38.1.REG.089-100.1

Oxymatrine reduces neuropathic pain in diabetic mice through the p38 MAPK/NF-κB signaling pathway

Wei Jiang, Yong Yao and Cheng Chen*
Pain Department, The Central Hospital of Enshi, 158 Wuyang Avenue, Enshi City, Hubei Province, China

Abstract: Diabetic neuropathic pain (DNP) is the most painful complications of diabetes. Oxymatrine (OMT) is one of the main components of Bitter Ginseng (radix *Sophorae flavescentis*) and has therapeutic effects on many secondary complications associated with diabetes mellitus; however, whether it improves DNP is unknown. The DNP mice model was induced by injecting streptozotocin (STZ), and alterations in body weight, blood glucose, mechanical nociceptive and thermal nociceptive sensitivity were measured over an 8-week period to determine the induction time needed to create DNP mice. OMT were injected intraperitoneally to see how different doses of OMT affected DNP, oxidative and inflammatory responses in diabetic mice. 0.2μg/kg p38 MAPK/NF-κB pathway agonist (P79350) was administered to investigate the influence on the action of OMT and explore the potential mechanism by which OMT alleviates DNP in diabetic mice. The best induction time for generating DNP mice was 4 weeks after continuous injection of 50mg/kg STZ. In mice, OMT effectively attenuated DNP, inhibited oxidative stress and inflammation. OMT inhibited the phosphorylation of pathway proteins p38 and NF-κB p65. However, activating p38 MAPK/NF-κB signaling with P79350 greatly reduced the effect of OMT. OMT attenuates DNP in diabetic mice via inhibiting p38 MAPK/NF-κB signaling.

Keywords: Oxymatrine, diabetic neuropathic pain, diabetes, p38 MAPK/NF-κB pathway.

Submitted on 14-10-2024 – Revised on 15-11-2024 – Accepted on 20-11-2024

INTRODUCTION

Diabetes is becoming more common over the world as the economy grows and people's lifestyles evolve. Diabetes prevalence reached 10.5% globally in 2021, an increase of 74 million compared to 2019 and it is expected to reach 20% by 2045 (Saeedi *et al.*, 2019; Sun *et al.*, 2022a). Diabetic neuropathy is the most prevalent chronic complication of diabetes mellitus, accounting for up to 50% of cases; roughly 60% of people with neuropathy report neuropathic pain (Sun *et al.*, 2020). Diabetic neuropathic pain (DNP) is one of the most common serious complications of diabetes, even in pre-diabetes. DNP patients experience moderate to severe numbness, burning pain, and electric shock-like tingling in their distal limbs (Paisley and Serpell, 2017), which is often intolerable for the patient and causes sleep disorders and depression, weakening the patient's psychological and social functioning and imposing a significant economic burden on the patient's family and society (D'Amato *et al.*, 2016). DNP is more difficult and expensive to treat than other forms of neuropathic pain (Sadosky *et al.*, 2015). Currently, the primary therapeutic strategy for DNP is glycemic control, followed by the use of medications to treat pain symptoms, such as tricyclic antidepressants, 5-hydroxytryptamine and norepinephrine dual-channel reuptake inhibitors calcium ion blockers, anticonvulsant, nonsteroidal anti-inflammatory drugs and opioids, among others (Colloca *et al.*, 2017; Galicia-Garcia *et al.*, 2020; Schreiber *et al.*, 2015), which are frequently poorly therapeutic and intolerant by patients. Until date, there has been a shortage of safe and effective DNP treatment in the clinic, making it especially vital to create innovative DNP medicines and identify new therapeutic targets.

Bitter Ginseng (radix *Sophorae flavescentis*) is a type of commonly used Chinese herbal medicine that has the effects of clearing heat and drying dampness, killing worms, and acting as a diuretic. It is primarily used to treat hot dysentery, blood in the stool, eczema, swollen and itchy pubic area, trichomoniasis, itchy skin, and scabies (Chen *et al.*, 2020a; Li *et al.*, 2021; Sun *et al.*, 2022b). Oxymatrine (OMT) is one of the main effective components of bitter ginseng. It is a double thick piperidine alkaloid and its chemical structure is $C_{30}H_{40}N_2O_6$. Modern pharmacological studies have shown that OMT has anti-inflammatory, antiviral, anti-tumor, immune system modulation and analgesic properties, among others. The clinical application of kuh-seng injection has been proven to have good analgesic effects on mild to moderate cancer pain, as well as a very good therapeutic effect on neuralgia produced by herpes zoster (Wang *et al.*, 2015). Furthermore, OMT not only reduced oxidized low-density lipoprotein damage to umbilical vein endothelial cells and apoptosis, but it also inhibited NLRP3 inflammasome-mediated cellular pyroptosis by activating the SIRT1/Nrf2 signaling pathway (Jin *et al.*, 2021), indicating that OMT protects the vascular endothelium and reduces diabetic vascular complications. OMT has been shown to decrease p65 and IκB

**Corresponding author:* e-mail: jiangwei000561@hotmail.com

phosphorylation, hindering NF-κB signaling. This reduces the generation of inflammatory components and alleviates inflammatory responses (Zhu *et al.*, 2020). OMT inhibits tumor cell proliferation, EMT, and death by regulating the NF-κB pathway (Halim *et al.*, 2019). These investigations demonstrate that OMT may modulate the NF-κB signaling pathway to alleviate DNP in mice; however the particular mechanism remains unclear.

In this study, a DNP mouse model was created by administering streptozotocin (STZ). STZ is the most selected chemical drug to induce diabetes model, which has specific damage to islet β cells and can cause diabetes (Akinlade *et al.*, 2021). Mice were injected intraperitoneally with varying concentrations of OMT to study its impacts on body weight, fasting blood glucose, mechanical and thermal nociceptive sensitivity and p38 MAPK/NF-κB pathway protein expression in DNP mice. Then, the p38 MAPK agonist P79350 was injected into the mice and the changes of DNP, oxidative stress index content in serum, as well as the number of microglia in spinal cord tissues and inflammatory infiltration, were observed to investigate the potential mechanism of OMT in regulating DNP in diabetic mice. This research will give a new theoretical basis for OMT in the treatment of DNP, while also promoting the use and popularization of TCM in the field of diabetes.

MATERIALS AND METHODS

Animal feeding

The Central Hospital of Enshi Ethics Committee granted approval and consent for all animal studies in this study. C57BL/6 mice aged 6 to 8 weeks were purchased from the Shanghai Research Center for Innovation of Traditional Chinese Medicine. Mice were housed at 23±2°C with a 12-hour light/12-hour dark cycle, 50%~60% humidity, and free access to water and food.

DNP model construction

Mice were acclimatized for one week before being assigned at random into two groups: Sham (n=6) and STZ (n=16). Mice in the Sham group received an intraperitoneal injection of 5mL/kg citrate buffer, while mice in the STZ group received an intraperitoneal injection of 50 mg/kg STZ (S0130, Sigma-Aldrich, Northbrook, IL, USA), both of which were administered continuously for 5 days. Mice were then assessed for body weight, fasting blood glucose, mechanical nociceptive and thermal nociceptive changes once a week for a total of 8 weeks.

6 mice in the Sham group and 12 mice in the STZ group were taken. Body weight changes were recorded, and mice's fasting blood glucose was monitored with a glucometer (Roche, Basel, Switzerland). Mechanical nociceptive: The mice adapted to the environment three days before the test and were placed in a transparent glass cover for 30 min every day to reduce measurement errors. The Up and Down approach was used to determine the Paw withdrawal threshold (PWT) of the mice. After the mice were placed until quiet, the lowest-intensity Von Frey Filaments (Yuyan instruments, Shanghai, China) were first used to vertically stimulate the center of the plantar surface of the left and right hind paws of mice, avoiding insensitive areas such as the foot pads. The cilia were flexed into an S-shape by applying a moderate force for a duration of 1~3 s, with a 5 min interval between stimulation sessions. Positive reactions were characterized as the existence of a quick foot-retraction reflex, licking response, or toe dorsal extension response in the mice's hind paw; otherwise, they were classified as negative. If the mice did not respond positively to the selected filaments, Von Frey filaments with a higher intensity were used for stimulation; if the response was positive, Von Frey filaments with a low intensity were used to stimulate until the mice did not produce a withdrawal avoidance response and PWT was recorded. Each mouse experiment was repeated three times and the average value was taken.

Heat nociceptive test: Following three days of acclimatization training, mice were assessed for heat nociceptive using the Hargreaves Test. The room temperature was maintained at 24°C and the surrounding environment was kept quiet. The mice were placed on a thermally conductive glass plate to allow the mice to adapt for more than 30 min. After being quiet, the right plantar area of the mice was irradiated with an infrared light source located below the mice and the heat was generated by focusing the light and causing pain. The irradiation was stopped until the right foot of the mice was lifted, shaken or licked. The longest irradiation time was 20s. If it exceeded 20s, the irradiation would be stopped automatically to avoid scalding the skin of the mice. The right plantar was irradiated once every 5 min, and the stimulation interval was 5 min. The paw withdrawal latency (PWL), also known as the thermal pain threshold, was measured 5 times continuously and averaged.

STZ-induced diabetic mice can develop characteristic hyperglycemic and neuropathic pain sensations within 4 weeks, which is consistent with the clinical aspects of DNP (Anjaneyulu and Chopra, 2003).

OMT's effects on DNP models and mechanisms of action

To study the influence of OMT on the DNP model, mice were divided into five groups: Sham (n=6), DNP model (intraperitoneal injection of 50 mg/kg STZ, four weeks of consecutive injections, n=4), DNP+OMT-40 (model + intraperitoneal injection of 40mg/kg OMT, n=4), DNP+OMT-80 (model + intraperitoneal injection of 80

mg/kg OMT, n=4), and DNP+OMT-160 (model + intraperitoneal injection of 160 mg/kg OMT, n=4). OMT was injected for one week beginning in week four, with the index test done in week six. Body weight, fasting blood glucose, mechanical nociceptive, and thermal nociceptive changes were recorded during the mice's feeding time.

Mice were separated into four groups: Sham (n=6), DNP model (n=6), DNP+OMT (model + 160mg/kg OMT, n=6), and DNP+OMT+P79350 (model + 160 mg/kg OMT + 0.2 μg/kg P79350, n=6) to investigate the OMT regulation mechanism. P79350 functions as a p38 MAPK agonist. Indicator tests were performed at week 6.

Acquiring samples of mice serum and spinal cord tissue

At week 6, the mice were executed, and blood samples were collected from the tail vein, centrifuged, and serum extracted. After anesthesia with 1% sodium pentobarbital, the mice were placed on top of the operating table, and the hearts of the mice were exposed with surgical instruments such as scissors and hemostatic forceps. A small incision was made in the right auricle with scissors, and an infusion needle was introduced at the left ventricular region for PBS perfusion until all the blood flowed out of the mice (the mice's liver appeared white). The mice's paraspinal muscles and tissues were separated to expose the spinal canal, which was then cut along the ventral midline of the spine with scissors, and the undamaged spinal cord tissue was taken and placed in a freezing tube. Both serum and spinal cord tissues were kept in a refrigerator at -80ºC and used for the oxidative stress kit assay, ELISA and Western blot investigations, respectively. The spinal cord tissue used for immunofluorescence and HE experiments was perfused with 4% paraformaldehyde solution using an infuser after the blood flow was completed and the perfusion was stopped when the rats' whole body muscle trembled, the limbs' muscle tone increased, and the phenomenon of stiffness and extension appeared, and the rats' spinal cord tissues were carefully removed and preserved in 4% paraformaldehyde solution.

Oxidative stress detection

Malondialdehyde (MDA, BC0025, Solarbio), superoxide dismutase (SOD, BC0175, Solarbio), catalase (CAT, BC0205, Solarbio), and glutathione (GSH, BC1175, Solarbio) were detected using the appropriate kits. The corresponding reagents were prepared according to the instructions manual and mixed separately with mice serum, after which the absorbance of each group of samples at different wavelengths was measured using an enzyme labeling apparatus, and the relative levels of MDA, SOD, CAT, and GSH in mice serum were calculated. MDA, for example, reacts with thiobarbituric acid in acidic and high-temperature conditions to produce brownish-red trimethine with an absorbance of 532 nm. SOD may scavenge O^{2+} and prevent the O^{2+} reduction of nitrogen blue tetrazolium, resulting in blue formazan with an absorption wavelength of 560 nm. CAT decomposes H_2O_2 and reduces its absorbance strength at 240 nm. GSH can form a compound with DTNB (Ellman's Reagent), which has a distinct absorption peak at 412 nm.

Spinal cord tissue section preparation

The spinal cord tissue was removed from a 4% paraformaldehyde solution, dehydrated, and dried to create a paraffin block. It was then cut into 4 μm thick paraffin slices using a slicing machine, slowly placed in a 40ºC water bath, carefully salvaged using well-marked slides, baked at high temperatures to ensure a tight fit on the slides, and stored in a specimen box for future use.

ELISA for inflammatory factors

Spinal cord tissue was assessed using the ELISA (enzyme-linked immunosorbent test) method. Spinal cord tissue was combined with lysate, homogenized, and centrifuged to extract the supernatant. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) concentrations were measured using Beyotime's PI301 and PT512 kits, following the instructions. 100μL of sample and 100μL of analytical buffer were added to the plate and incubated at 37ºC for 2 h. Next, 100μL of biotinylated antibody was added to the plate and incubated at 37ºC for 1.5 h. To begin, add 100μL of affinity hormone-horseradish peroxidase marker and incubate at 37ºC for 20 min. Next, add 100μL of TMB color development solution and incubate for 20 min away from light. Finally, add 50μL of termination solution to end the reaction. The absorbance of the samples at 450 nm was determined with an enzyme marker. To establish a standard curve, the absorbance value was utilized as the vertical coordinate and the concentration of the standard as the horizontal coordinate. The concentrations of IL-1β and TNF-α were then calculated based on the curve.

Immunofluorescence detection

Spinal cord tissue sections were treated with 0.1% Triton X-100 permeabilization and 2% BSA closure. This was followed by an overnight incubation at 4ºC with rabbit anti-Iba-1 (ab178847, 1:100; Abcam, Waltham, MA, USA). The nuclei were rinsed with PBS before incubating with FITC-labeled sheep anti-rabbit IgG (ab205718, 1:2000) at 37ºC for 1 h. The nuclei were stained with DAPI for 5 min, then washed with PBS, and the expression level of Iba-1 in spinal cord tissues was observed using a fluorescence inverted microscope after adding an anti-fluorescence quencher.

HE staining

Spinal cord tissue sections were dewaxed in xylene (1330-20-7, Nanjing Reagent, Nanjing, China), rinsed in water, gradient dehydrated with ethanol, and stained with hematoxylin (C0107, Beyotime, Shanghai, China) for 3~5

min. The sections were washed in running water, differentiated briefly in 1% acidic alcohol (containing 70% hydrochloric acid), and rinsed in running water. Blue for a few moments with 1% ammonia solution, then rinse. Dip in 0.5% eosin (G1100, Solarbio) for 3 min, then rinse under running water. Gradient dehydration in ethanol (70% ethanol for 1 min, remove and shake dry, 80% ethanol for 1 min, remove and shake dry, 95% ethanol for 10 min, and three times in anhydrous ethanol for 20 min each), followed by three xylene clear treatments (20 min each). Finally, the slices were sealed using neutral gum (G8590 from Solarbio). Pathologic alterations to the spinal cord were identified under a microscope.

Western blot

Spinal cord tissues were treated in RIPA lysate (E-BC-R327, Elabscience, Wuhan, China) for 10 min, then total proteins were extracted and concentrations measured. Proteins were electrophoretically separated on SDS-PAGE gels before being transferred to a PVDF membrane and sealed for 1 h. The membrane was co-incubated with the corresponding primary antibody at 4°C overnight, and then incubated with horseradish peroxidase-coupled secondary antibody (1:2000, ab6721, Abcam) for 1h at room temperature. The color was revealed through dropwise color development of the chemiluminescent solution using ECL (P0018S, Beyotime). Finally, image analysis software was utilized for detection and quantification. This work used the following primary antibodies: p-p38 (1:1000, ab4822, Abcam), p38 (1:1000, ab316937, Abcam), p-NF-κB p65 (1:1000, ab76302, Abcam), NF-κB p65 (1:1000, ab32536, Abcam), and the internal reference GADPH (1:2500, ab9485, Abcam).

Ethical approval

The Central Hospital of Enshi Ethics Committee granted approval and consent for all animal studies in this study (No. 2023-083-005).

STATISTICAL ANALYSIS

Each experiment was performed at least three times, and the results were analyzed using one-way ANOVA and Student's *t*-tests in SPSS 26.0 and plotted in Graphpad Prism 9.0. **P*<0.05 indicates a significant difference. Data is presented as mean ± standard deviation of means.

RESULTS

STZ intraperitoneal injection induces DNP in mice

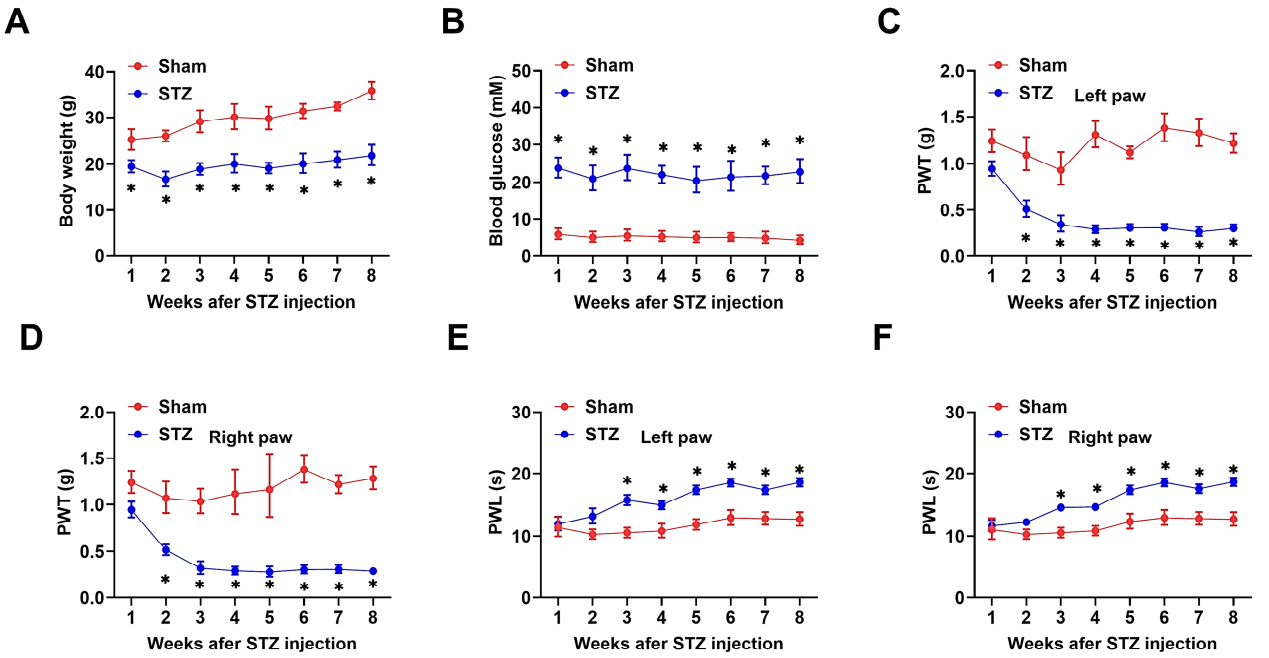
Throughout the experiment, the mice in the Sham group had smooth and soft fur, as well as normal daily water intake and urine output, whereas the mice in the STZ group developed dull and rough fur and significantly increased their dietary water intake and urine output. Compared to the Sham group, the mice in the STZ group lost body weight and gained blood glucose during 1-8 weeks of feeding, and the mice's fasting blood glucose was always greater than 8.0 mM (figs. 1A-1B), indicating that STZ induced the establishment of a diabetes mellitus model in mice (Kennard *et al.*, 2021). After mice were continuously injected with STZ (50 mg/kg) for 5 d, PWT began to decrease significantly in the second week and continued to stabilize in the fourth week (figs. 1C-1D); PWL increased significantly in the third week, and the mice's behavior gradually stabilized after 4 weeks (figs. 1E-1F). These findings indicated that the diabetic DNP mice model was successfully established after the mice were laparoscopically injected with STZ, and that the diabetic mice lost weight, increased blood glucose and demonstrated significant hypersensitivity to mechanical stimuli over time. Furthermore, the hind limbs of mice showed hypersensitivity to thermal stimuli after STZ induction, which may be due to the reduced density of nerve fibers within the epidermis of the mice's foot pads, implying that the mice developed neuropathy following SZT induction, which is consistent with the findings of Chen *et al.* (2022). All subsequent mice DNP models were created by injecting 50 mg/kg of STZ for four weeks.

OMT attenuates neuropathic pain in diabetic mice

To investigate the impacts of OMT on DNP in diabetic mice, mice were intraperitoneally injected with 40 mg/kg, 80 mg/kg, and 160 mg/kg of OMT for one week at the fourth week of STZ induction, and changes in body weight, blood glucose, PWT and PWL were observed in each group at the sixth week. As shown in figs. 2A-2F, OMT action significantly weakened the induced effect of STZ on mice, mice body weight regained (figs. 2A), blood glucose decreased (figs. 2B), and mice hind limb PWT (figs. 2C-2D) and PWL (figs. 2E-2F) increased and decreased, respectively, and the effect of OMT action was gradually significant with increasing concentration, implying that DNP was improved in mice. It was proven that OMT could effectively reduce NDP in diabetic mice.

OMT inhibits oxidative stress in diabetic mice

Oxidative stress (OS), defined as an imbalance between oxidative and antioxidant effects *in vivo*, has an important role in the etiology of a variety of diseases, including neurological disorders, spinal cord injury and DNP. To investigate further indications of worsening of oxidative stress injury, we used various kits to detect changes in the levels of the oxidative indicator MDA as well as the antioxidant indicators SOD, CAT and GSH in mice serum. As shown in figs. 3A-3D, the serum MDA (fig. 3A) content of DNP mice was significantly higher than that of the Sham group, whereas the SOD (fig. 3B), CAT (fig. 3C), and GSH (fig. 3D) contents were significantly lower, indicating that STZ induced oxidative stress injury in mice, which could be an important reason for the development of DNP in diabetic mice. The oxidative stress phenomena in diabetic mice significantly weakened as the concentration of OMT injections increased.



A The mice were randomly separated into Sham and 50 mg/kg STZ-induced groups, and the body weight changes of each group were recorded over an 8-week period of feeding.
B Glucometers were used to measure changes in mice's fasting blood glucose levels during the feeding period.
C-D The mechanical nociceptive sensitivity of mice during feeding was measured using an electronic Von Frey analgesometer.
E-F The Hargreaves measure was used to measure mice's thermal nociceptive levels.

Fig. 1: STZ-induced diabetic neuropathic pain in mice.

OMT inhibits the inflammatory response in diabetic mice

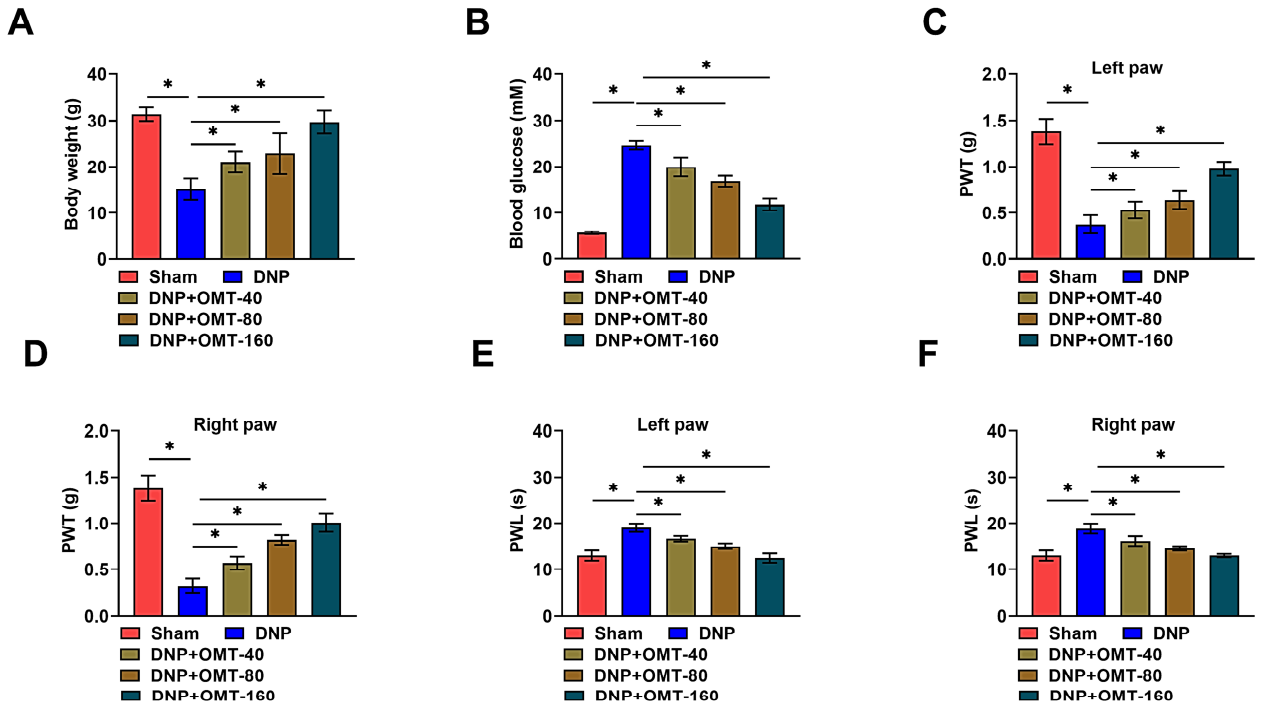
In addition to oxidative stress, the DNP process involves an inflammatory response induced by hyperglycemia. fig. 4A-4B show that DNP mice had significantly higher levels of inflammatory factors IL-1β and TNF-α in their spinal cord tissues compared to the control group. These levels gradually decreased as the concentration of OMT injections increased. The most significant inhibitory effect of OMT was at 160 mg/kg. In the diseased state, microglia caused inflammatory responses in the central nervous system, exacerbating nerve damage (Savage *et al.*, 2019). Immunofluorescence measured changes in the expression of the microglia marker Iba-1 in spinal cord tissues consistent with inflammatory factors (fig. 4C).

Which is consistent with previous findings. HE staining of spinal cord tissue sections revealed that the DNP group had structural destruction of the spinal cord, disorganized tissue arrangement, formation of cavities and infiltration of a large number of inflammatory cells in the damaged area, which could be effectively improved by OMT, especially after treatment with 160 mg/kg OMT, the spinal cord tissue tended to be intact, with a normal structural arrangement, and there was almost no edema and infiltration of inflammatory cells (fig. 4D). These assays indicated that OMT could effectively inhibit the inflammatory response in diabetic mice and 160 mg/kg OMT was subsequently selected for subsequent mechanism studies.

OMT attenuates DNP in diabetic mice via the p38 MAPK/NF-κB signaling pathway

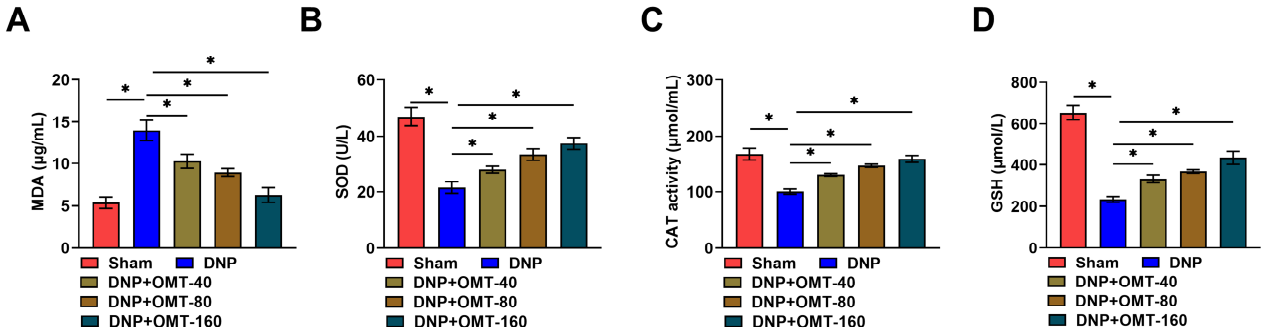
We injected mice with 0.2μg/kg P79350 and evaluated the phosphorylation levels of pathway proteins p38 and NF-κB p65 in spinal cord tissues of each group using Western blot. fig. 5A-5C show the level of p-p38 and p-NF-κB p65 was significantly elevated in the DNP group, indicating that STZ activated the p38 MAPK/NF-κB signaling pathway, which was then inhibited by OMT treatment. However, the agonist P79350 significantly reduced the effect of OMT and reactivated the pathway. Following measurements of body weight, blood glucose, and hindlimb nociceptive behaviors revealed that P79350 partially reversed the effects of 160 mg/kg OMT on DNP mice, which were exacerbated by decreased body weight (fig. 5D), increased blood glucose (fig. 5E), enhanced mechanical nociceptive sensitivity (fig. 5F-5G), and decreased thermal nociceptive sensitivity (fig. 5H-5I). The study found that OMT reduced DNP levels in mice by suppressing the p38 MAPK/NF-κB pathway.

OMT inhibits oxidative stress and inflammatory responses in diabetic mice through the p38 MAPK/NF-κB signaling pathway



A The DNP mice model was constructed using 50mg/kg STZ induction for 4 weeks, then 40mg/kg, 80mg/kg and 160mg/kg of OMT were intraperitoneally injected for 1 week. The effect of OMT on mice body weight was examined in the sixth week.
B The effect of OMT on mice's fasting blood glucose was measured using a glucometer.
C-D The impact of OMT on mechanical nociceptive sensitivity in mice was investigated.
E-F The effect of OMT on heat nociceptive in mice was investigated using the Hargreaves Test.

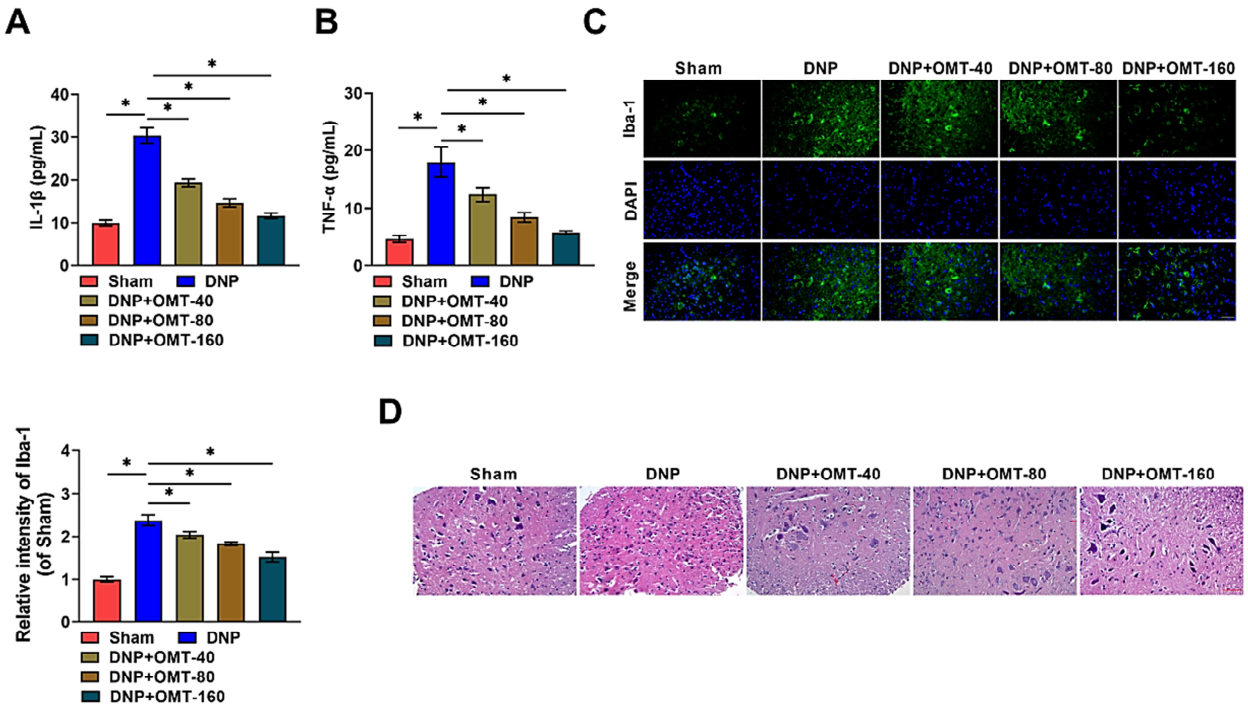
Fig. 2: OMT reduces neuropathic pain in diabetic mice



A The mice were killed at the end of week 6, and blood samples were drawn from the tail vein and centrifuged to extract serum. The effect of OMT on the content of inflammatory factors in mice serum was observed. The kit measures the level of oxidized MDA in serum.
B-D Kits were used to determine the amounts of antioxidants SOD, CAT, and GSH in serum.

Fig. 3: OMT reduces oxidative stress in diabetic mice

To verify whether OMT mediated the p38 MAPK/NF-κB pathway to inhibit oxidative stress and inflammatory responses in diabetic mice, we examined changes in serum oxidative/antioxidant markers and inflammatory factors, as well as microglia and inflammatory cell infiltration in the spinal cord tissue sections. Fig. 6A-6D show that activating the p38 MAPK/NF-κB pathway dramatically raised MDA content in mice serum. However, measuring SOD, CAT and GSH content indicated the opposite trend. The agonist P79350 increased the inflammatory markers IL-1β and TNF-α, as well as the amount of microglia in mice's spinal cord tissues (fig. 6E-6G). Pathological damage in tissue sections, including inflammatory cell infiltration and edema, was linked to the p38 MAPK/NF-κB signaling pathway (fig. 6H). The results confirmed that OMT mediated the p38 MAPK/NF-κB signaling pathway to inhibit oxidative stress and inflammatory responses in diabetic mice.



A-B ELISA was used to measure the expression of inflammatory factors IL-1β and TNF-α in spinal cord tissues treated with various doses of OMT.
C Immunofluorescence was used to detect the expression of the microglial cell marker Iba-1 in spinal cord samples at various OMT treatment dosages.
D HE staining detects inflammatory cell infiltration in spinal cord tissues at various OMT treatment doses.

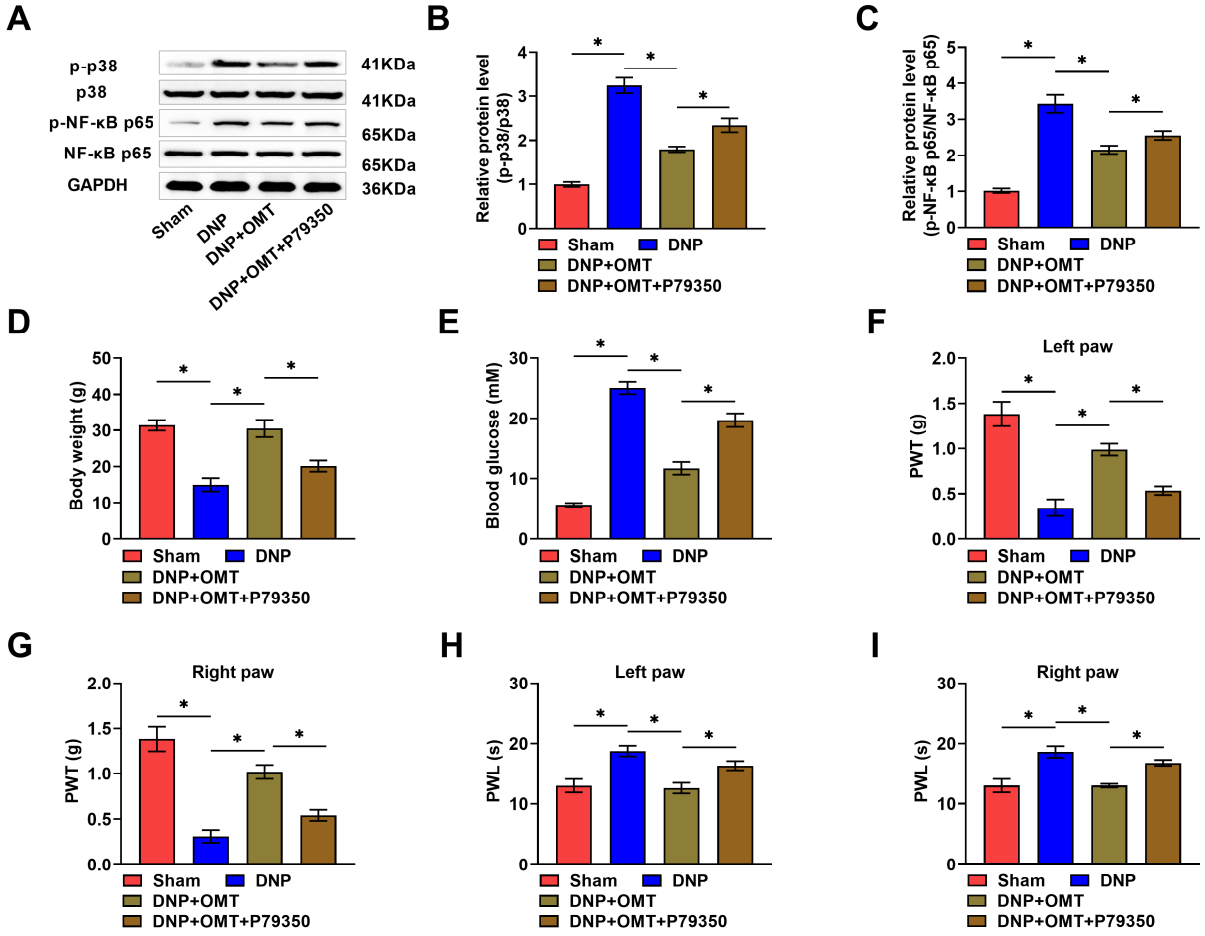
Fig. 4: OMT reduces inflammatory responses in diabetic mice.

DISCUSSION

Traditional Chinese medicine (TCM) has been utilized as a popular therapy for diabetes-related problems. In our work, OMT (particularly the high dosage of OMT, 160mg/kg) significantly increased the growth of DNP mice, lowered fasting blood glucose, and boosted mechanical nociceptive sensitivity in mice. In addition, OMT injections reduced oxidant MDA in mice serum and inflammatory factors (IL-1β, TNF-α) in spinal cord tissues. However, antioxidants SOD, CAT and GSH were gradually up-regulated.

Microglia marker Iba-1 and inflammatory cell infiltration increased in spinal cord tissues. These findings give a scientific foundation for treating diabetic DNP with OMT.

Oxidative stress is a biological state in which there is an imbalance between oxidative and antioxidant effects in the body, and pro-oxidant chemicals such as reactive oxygen/nitrogen (ROS / NOS) exceed the loading capacity of the antioxidant system. Oxidative stress is capable of generating changes in the chemical structure of cellular components such as lipids and proteins (Li *et al.*, 2023) and is involved in and plays a critical part in the pathogenesis of a number of conditions such as neurological disorders, spinal cord injury and neuropathic pain (Teleanu *et al.*, 2022). Oxidative stress is currently recognized as one of the major causes of many neuropathic pains, such as diabetic peripheral neuropathy and post-chemotherapy peripheral neuropathy (Ye *et al.*, 2022). Oxidative stress can cause neuropathy through a variety of mechanisms, including biological failure, loss of antioxidant defenses, biomolecular damage, microtubule rupture, ion channel activation, demyelination, neuroinflammation, neuronal death due to mitotic damage and apoptosis (Lin *et al.*, 2023; Teixeira-Santos *et al.*, 2020). Among them, oxidative stress-induced mitochondrial dysfunction and neuronal damage are thought to be important in the development of neuropathic pain (Singh *et al.*, 2019). The opening of the mitochondrial permeability transition pore (mPTP), mitochondrial swelling and vacuolization lead to mitochondrial dysfunction, which further triggers Ca^{2+} release, cysteine asparaginase activation, and neuronal apoptosis (Godoy *et al.*, 2021; Neginskaya *et al.*, 2021). Neuronal apoptosis stimulates microglia to release pro-inflammatory mediators and growth factors in the area of injury, sensitizing peripheral nerves, resulting in spontaneous discharges and increased excitability (Fricker *et al.*, 2018; Wang *et al.*, 2022). One of the reasons why platinum-based chemotherapeutic agents, such as cisplatin



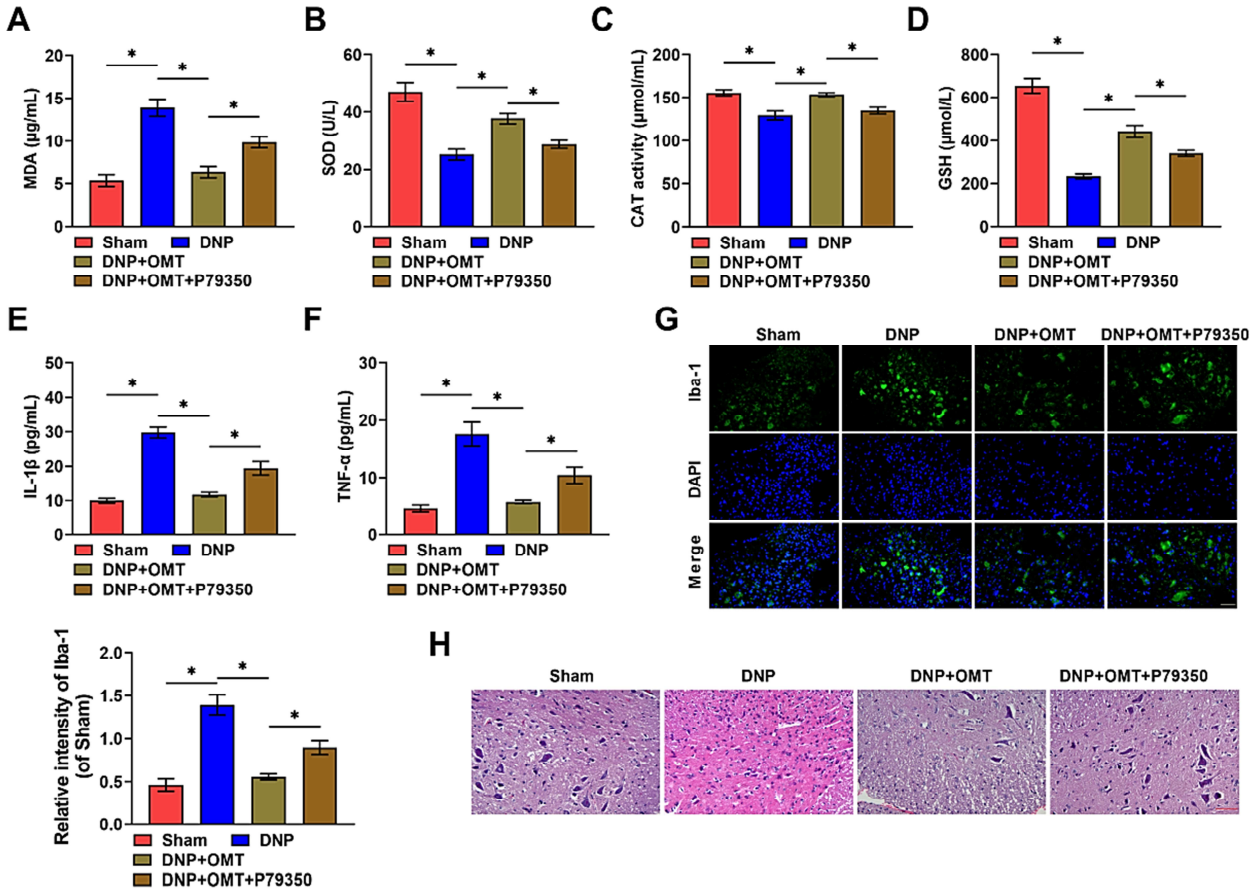
A-C P79350 was injected into the tail vein at 0.2μg/kg body weight. Western blotting was used to detect the level of p38 MAPK/NF-κB pathway proteins in spinal cord tissues.
D Mice's body weights were recorded.
E The fasting blood glucose levels in the tail veins of mice were measured using a glucometer.
F-G Mechanical nociceptive was measured in mice using electronic Von Frey analgesia.
H-I Thermal nociceptive in mice utilizing the Hargreaves Test.

Fig. 5: In diabetic mice, OMT reduces neuropathic pain by regulating the p38 MAPK/NF-κB pathway.

and oxaliplatin, cause neuropathic pain is their ability to affect mitochondrial DNA, causing mitochondrial dysfunction, which eventually damages sensory neurons, resulting in neuropathic pain characterized by nociceptive sensitization and nociceptive abnormalities (Dong *et al.*, 2022; Quintao *et al.*, 2019). In the current work, OMT reduced oxidative stress injury in mice (lower MDA content and increased SOD, CAT, and GSH content), and diabetic mice demonstrated decreased sensitivity to mechanical stimuli. This demonstrates that oxidative stress is a significant factor in the development of DNP.

In addition to oxidative stress, the inflammatory response plays a crucial part in the induction of diabetic DNP. Following a spinal cord injury, an inflammatory response occurs in the injured area, with neutrophils and macrophages immediately activating and recruiting to the lesion site (Freyermuth-Trujillo *et al.*, 2022; Hellenbrand *et al.*, 2021; Sterner and Sterner, 2022). As a result, these immune cells create and secrete a range of cytokines, which govern nerve cell regeneration (Ma *et al.*, 2019).

Massive immune cell infiltration causes secondary damage such as inflammation, glutamate excitotoxicity, apoptosis, pyroptosis, and free radical-induced cell death, all of which encourage growth of the damaged brain tissue area and worsen neurological impairments and prognosis (Wang *et al.*, 2019). Further microglia activation, infiltration of neutrophils, monocytes/macrophages and other cells and rapid induction of cascade reactions (release of ROS and pro-inflammatory cytokines, etc.) are then promoted (Jiang *et al.*, 2018), severely affecting functional recovery of the spinal cord as well as the organism's motor and sensory functions. Early inflammation is both beneficial and detrimental, calling on a large number of cells to mount an anti-



A-D Mice were given 0.2μg/kg body weight of P79350 in the tail vein. The kit detected changes in the expression of oxidative stress-related markers in serum.
E-F The level of inflammatory factors IL-1β and TNF-α in spinal cord tissues was measured using ELISA.
G Immunofluorescence is used to detect the expression of the microglial cell marker Iba-1 in spinal cord samples.
H The effect of P79350 on inflammatory cell infiltration in spinal cord tissues was determined using HE staining.

Fig. 6: In diabetic mice, OMT inhibits oxidative stress and inflammation by regulating the p38 MAPK/NF-κB pathway

inflammatory response. Numerous studies have shown that inflammation spreads to the surrounding tissues, causing cell death and the release of substances that inhibit spontaneous regeneration and functional recovery of the tissues (DiSabato *et al.*, 2016; Jurcau and Simion, 2021). The release of a large amount of inflammatory factors can cause an "inflammatory factor storm," which triggers a series of secondary damage reactions, induces immune damage and exacerbates spinal cord tissue damage (Anjum *et al.*, 2020; Khaing *et al.*, 2023). As a result, reducing the inflammatory response, limiting the manufacture and release of inflammatory proteins, and improving the stability of cells and lysosomal membranes all play key roles in spinal cord injury treatment. Mice treated with STZ for 4 weeks showed significantly higher levels of IL-1β and TNF-α inflammatory factors, microglia marker protein Iba-1 and inflammatory cell infiltration in spinal cord tissues compared to the Sham group, suggesting that oxidative stress injury occurs in the spinal cord of mice, which was alleviated after treatment with OMT.

MAPK p38 regulates NF-κB transcriptional activity in cells via acetylating p65 (Chen *et al.*, 2020b). The NF-κB family of proteins, comprising RelA (p65), RelB, Rel, p105/p50, and p100/p52, is involved in various biological processes such as cell proliferation, differentiation, apoptosis, senescence, inflammation and immunological response (Alharbi *et al.*, 2021). NF-κB protein activity is regulated by IκB protein, and activation of IκB kinase phosphorylates and degrades IκB protein, resulting in nuclear translocation of free NF-κB protein from cytoplasm to nucleus. This activates a series of cytokine transcriptional processes (Gong *et al.*, 2023), including pro-inflammatory factors such as MMPs, iNOS, IL-1β, TNF-α, etc. (Cavalcanti *et al.*, 2021; Tang *et al.*, 2021), which are closely related to DNP and other diabetic complications (Entezari *et al.*, 2022; Tang and Yiu, 2020). Studies have shown that in the central nervous system, activation of NF-κB and MAPK pathways can promote the production of inflammatory mediators (He *et al.*, 2020). Sec-O-glucosylhamaudol reduces pro-

inflammatory cytokines and relieves neuropathic pain in rats by inhibiting JNK/p38 MAPK and NF-κB signaling pathways (Oh *et al.*, 2021). It indicates that p38 MAPK/NF-κB signaling pathways play an important role in neuroinflammation and pain. Western blot analysis revealed that STZ-induced diabetic DNP in mice promoted phosphorylation of p38 and NF-κB p65 proteins, activating the p38 MAPK/NF-κB pathway. While OMT injection inhibited this phenomenon, P79350 attenuated the inhibitory effect, indicating that OMT can alleviate diabetic DNP in mice by blocking p38 MAPK/NF-κB signaling.

CONCLUSION

In summary, DNP rats had dramatically enhanced fasting blood glucose and mechanical nociceptive sensitivity. OMT can reduce DNP in diabetic mice by inhibiting the p38 MAPK/NF-κB pathway-mediated oxidative stress and inflammatory response. This study provides a theoretical basis for the application of OMT in DNP. OMT may be an effective treatment for DNP; however, the mechanism of its analgesia is not entirely understood, and there are still some unknown channels related to DNP, which are worthy of further exploration. Using multi-level studies at the molecular, cellular and animal levels, we will predict the drug targets of OMT built a network of interactions between active ingredients and proteins, screen the key genes of OMT in the treatment of DNP by network topology analysis and identify signaling pathways involved in key nodes that directly act on analgesia, providing a more comprehensive understanding of OMT in the treatment of DNP. Furthermore, most of the existing OMT experiments only stay at the level of cells and animals and do not enter the stage of human experiments, lacking clinical verification. The safety and effectiveness of OMT in clinical applications need to be further evaluated in the future. Therefore, clinical value-oriented pharmacodynamic studies are carried out to explore the safety and effectiveness of OMT and promote mutual complementation and coordinated development of OMT and Western medicine.

REFERENCES

Akinlade OM, Owoyele BV and Soladoye AO (2021). Streptozotocin-induced type 1 and 2 diabetes in rodents: A model for studying diabetic cardiac autonomic neuropathy. *Afr. Health Sci.,* **21**: 719-727.

Alharbi KS, Fuloria NK, Fuloria S, Rahman SB, Al-Malki WH, Javed Shaikh MA, Thangavelu L, Singh SK, Rama Raju Allam VS, Jha NK, Chellappan DK, Dua K and Gupta G (2021). Nuclear factor-kappa B and its role in inflammatory lung disease. *Chem. Biol. Interact.,* **345**: 109568.

Anjaneyulu M and Chopra K (2003). Quercetin, a bioflavonoid, attenuates thermal hyperalgesia in a mouse model of diabetic neuropathic pain. *Prog. Neuropsychopharmacol. Biol. Psychiatry,* **27**: 1001-1005.

Anjum A, Yazid MD, Fauzi Daud M, Idris J, Ng AMH, Selvi Naicker A, Ismail OHR, Athi Kumar RK and Lokanathan Y (2020). Spinal cord injury: Pathophysiology, multimolecular interactions and underlying recovery mechanisms. *Int. J. Mol Sci.,* 21(20): 7533.

Cavalcanti MRM, Passos FRS, Monteiro BS, Gandhi SR, Heimfarth L, Lima BS, Nascimento YM, Duarte MC, Araujo AAS, Menezes IRA, Coutinho HDM, Zengin G, Ceylan R, Aktumsek A, Quintans-Junior LJ and Quintans JSS (2021). HPLC-DAD-UV analysis, anti-inflammatory and anti-neuropathic effects of methanolic extract of *Sideritis bilgeriana* (Lamiaceae) by NF-kappaB, TNF-alpha, IL-1beta and IL-6 involvement. *J. Ethnopharmacol.,* **265**: 113338.

Chen M, Ding Y and Tong Z (2020a). Efficacy and safety of *Sophora flavescens* (Kushen) based traditional chinese medicine in the treatment of ulcerative colitis: Clinical evidence and potential mechanisms. *Front. Pharmacol.,* **11**: 603476.

Chen SP, Su CH, Huang-Liu R, Lee MW, Chiang CY, Chen WY, Chen CJ, Wu SW and Kuan YH (2020b). Protective effect of nerolidol on lipopolysaccharide-induced acute lung injury through the inhibition of NF-κB activation by the reduction of p38 MAPK and JNK phosphorylation. *J. Funct. Foods,* **69**: 103943.

Chen W, Wang X, Sun Q, Zhang Y, Liu J, Hu T, Wu W, Wei C, Liu M, Ding Y, Liu D, Chong Y, Wang P, Zhu H, Cui W, Zhang J, Li Q and Yang F (2022). The upregulation of NLRP3 inflammasome in dorsal root ganglion by ten-eleven translocation methylcytosine dioxygenase 2 (TET2) contributed to diabetic neuropathic pain in mice. *J. Neuroinflammation,* **19**: 302.

Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, Freeman R, Truini A, Attal N, Finnerup N B, Eccleston C, Kalso E, Bennett D L, Dworkin RH and Raja SN (2017). Neuropathic pain. *Nat. Rev. Dis. Primers,* **3**: 17002.

D'Amato C, Morganti R, Greco C, Di Gennaro F, Cacciotti L, Longo S, Mataluni G, Lauro D, Marfia G A and Spallone V (2016). Diabetic peripheral neuropathic pain is a stronger predictor of depression than other diabetic complications and comorbidities. *Diab. Vasc. Dis. Res.,* **13**: 418-428.

DiSabato DJ, Quan N and Godbout JP (2016). Neuroinflammation: The devil is in the details. *J. Neurochem.,* **139**(Suppl 2): 136-153.

Dong ZB, Wang YJ, Cheng ML, Wang BJ, Lu H, Zhu H L, Liu L and Xie M (2022). 2-Bromopalmitate decreases spinal inflammation and attenuates oxaliplatin-induced neuropathic pain via reducing Drp1-mediated mitochondrial dysfunction. *PloS One*, **17**: e0275428.

Entezari M, Hashemi D, Taheriazam A, Zabolian A, Mohammadi S, Fakhri F, Hashemi M, Hushmandi K, Ashrafizadeh M, Zarrabi A, Ertas Y N, Mirzaei S and Samarghandian S (2022). AMPK signaling in diabetes mellitus, insulin resistance and diabetic complications: A pre-clinical and clinical investigation. *Biomed. Pharmacother.,* **146**: 112563.

Freyermuth-Trujillo X, Segura-Uribe JJ, Salgado-Ceballos H, Orozco-Barrios C E and Coyoy-Salgado A (2022). Inflammation: A target for treatment in spinal cord injury. *Cells*, **11**: 2692.

Fricker M, Tolkovsky AM, Borutaite V, Coleman M and Brown GC (2018). Neuronal cell death. *Physiol. Rev.,* **98**: 813-880.

Galicia-Garcia U, Jebari S, Larrea-Sebal A, Uribe K B, Siddiqi H, Ostolaza H, Benito-Vicente A and Martin C (2020). Statin treatment-induced development of type 2 diabetes: From clinical evidence to mechanistic insights. *Int. J. Mol. Sci.,* **21**: 4725.

Godoy JA, Rios JA, Picon-Pages P, Herrera-Fernandez V, Swaby B, Crepin G, Vicente R, Fernandez-Fernandez JM and Munoz FJ (2021). Mitostasis, calcium and free radicals in health, aging and neurodegeneration. *Biomolecules,* **11**: 1012.

Gong P, Jia HY, Li R, Ma Z, Si M, Qian C, Zhu F Q and Sheng-Yong L (2023). Downregulation of Nogo-B ameliorates cerebral ischemia/reperfusion injury in mice through regulating microglia polarization via TLR4/NF-kappaB pathway. *Neurochem Int.,* **167**: 105553.

Halim CE, Xinjing SL, Fan L, Bailey Vitarbo J, Arfuso F, Tan CH, Narula AS, Kumar AP, Sethi G and Ahn KS (2019). Anti-cancer effects of oxymatrine are mediated through multiple molecular mechanism(s) in tumor models. *Pharmacol. Res.,* **147**: 104327.

He Y, Ruganzu JB, Zheng Q, Wu X, Jin H, Peng X, Ding B, Lin C, Ji S, Ma Y and Yang W (2020). Silencing of LRP1 exacerbates inflammatory response via TLR4/NF-κB/MAPKs signaling pathways in APP/PS1 transgenic mice. *Mol. Neurobiol.,* **57**: 3727-3743.

Hellenbrand DJ, Quinn CM, Piper ZJ, Morehouse CN, Fixel JA and Hanna AS (2021). Inflammation after spinal cord injury: A review of the critical timeline of signaling cues and cellular infiltration. *J. Neuroinflammation,* **18**: 284.

Jiang X, Andjelkovic AV, Zhu L, Yang T, Bennett MVL, Chen J, Keep RF and Shi Y (2018). Blood-brain barrier dysfunction and recovery after ischemic stroke. *Prog. Neurobiol.,* **163-164**: 144-171.

Jin X, Fu W, Zhou J, Shuai N, Yang Y and Wang B (2021). Oxymatrine attenuates oxidized low-density lipoprotein-induced HUVEC injury by inhibiting NLRP3 inflammasome-mediated pyroptosis via the activation of the SIRT1/Nrf2 signaling pathway. *Int. J. Mol. Med.,* **48**: 187.

Jurcau A and Simion A (2021). Neuroinflammation in cerebral ischemia and ischemia/reperfusion injuries: From pathophysiology to therapeutic strategies. *Int. J. Mol. Sci.,* **23**: 14.

Kennard MR, Daniels Gatward LF, Roberts AG, White E RP, Nandi M and King AJF (2021). The use of mice in diabetes research: The impact of experimental protocols. *Diabet. Med.,* **38**: e14705.

Khaing ZZ, Chen JY, Safarians G, Ezubeik S, Pedroncelli N, Duquette RD, Prasse T and Seidlits SK (2023). Clinical trials targeting secondary damage after traumatic spinal cord injury. *Int. J. Mol. Sci.,* **24**: 3824.

Li H, Hung A, Li M, Lu L and Yang AWH (2021). Phytochemistry, pharmacodynamics and pharmacokinetics of a classic Chinese herbal formula Danggui Beimu Kushen Wan: A review. *Phytother. Res.,* **35**: 3673-3689.

Li J, Pan L, Pan W, Li N and Tang B (2023). Recent progress of oxidative stress associated biomarker detection. *Chem. Commun. (Camb).,* **59**: 7361-7374.

Lin Q, Li K, Chen Y, Xie J, Wu C, Cui C and Deng B (2023). Oxidative stress in diabetic peripheral neuropathy: Pathway and mechanism-based treatment. *Mol. Neurobiol.,* **60**: 4574-4594.

Ma JJ, Xu RJ, Qi SB, Wang F, Ma YX, Zhang HC, Xu J H, Qin XZ, Zhang HN, Liu CM, Li B, Chen JQ, Yang HL and Saijilafu (2019). Regulation of adult mammalian intrinsic axonal regeneration by NF-kappaB/STAT3 signaling cascade. *J. Cell Physiol.,* **234**: 22517-22528.

Neginskaya MA, Pavlov EV and Sheu SS (2021). Electrophysiological properties of the mitochondrial permeability transition pores: Channel diversity and disease implication. *Biochim. Biophys. Acta Bioenerg.,* **1862**: 148357.

Oh S H, Kim S W, Kim D J, Kim S H, Lim K J, Lee K and Jung KT (2021). Sec-O-glucosylhamaudol mitigates inflammatory processes and autophagy via p38/JNK MAPK signaling in a rat neuropathic pain model. *Korean J. Pain,* **34**: 405-416.

Paisley P and Serpell M (2017). Improving pain control in diabetic neuropathy. Practitioner. **261**, 23-26.

Quintao NLM, Santin JR, Stoeberl LC, Correa TP, Melato J and Costa R (2019). Pharmacological treatment of chemotherapy-induced neuropathic pain: PPARgamma agonists as a promising tool. *Front Neurosci.,* **13**: 907.

Sadosky A, Mardekian J, Parsons B, Hopps M, Bienen E J and Markman J (2015). Healthcare utilization and costs in diabetes relative to the clinical spectrum of painful diabetic peripheral neuropathy. *J. Diabetes Complications,* **29**: 212-217.

Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, Colagiuri S, Guariguata L, Motala A A, Ogurtsova K, Shaw J E, Bright D, Williams R and Committee IDFDA (2019). Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. *Diabetes Res. Clin. Pract.,* **157**: 107843.

Savage JC, Carrier M and Tremblay ME (2019). Morphology of microglia across contexts of health and disease. *Methods Mol. Biol.,* **2034**: 13-26.

Schreiber AK, Nones CF, Reis RC, Chichorro JG and Cunha JM (2015). Diabetic neuropathic pain: Physiopathology and treatment. *World J. Diabetes,* **6**: 432-444.

Singh A, Kukreti R, Saso L and Kukreti S (2019). Oxidative Stress: A key modulator in neuro-degenerative diseases. *Molecules,* **24**(8): 1583.

Sterner RC and Sterner RM (2022). Immune response following traumatic spinal cord injury: Pathophysiology and therapies. *Front. Immunol.,* **13**: 1084101.

Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, Stein C, Basit A, Chan JCN, Mbanya J C, Pavkov ME, Ramachandaran A, Wild SH, James S, Herman WH, Zhang P, Bommer C, Kuo S, Boyko EJ and Magliano DJ (2022a). IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res. Clin. Pract.,* **183**: 109119.

Sun J, Wang Y, Zhang X, Zhu S and He H (2020). Prevalence of peripheral neuropathy in patients with diabetes: A systematic review and meta-analysis. *Prim. Care Diabetes,* **14**: 435-444.

Sun P, Zhao W, Wang Q, Chen L, Sun K, Zhan Z and Wang J (2022b). Chemical diversity, biological activities and Traditional uses of and important Chinese herb sophora. *Phytomedicine,* **100**: 154054.

Tang G, Li S, Zhang C, Chen H, Wang N and Feng Y (2021). Clinical efficacies, underlying mechanisms and molecular targets of Chinese medicines for diabetic nephropathy treatment and management. *Acta Pharm. Sin. B.,* **11**: 2749-2767.

Tang SCW and Yiu WH (2020). Innate immunity in diabetic kidney disease. *Nat Rev Nephrol.,* **16**: 206-222.

Teixeira-Santos L, Albino-Teixeira A and Pinho D (2020). Neuroinflammation, oxidative stress and their interplay in neuropathic pain: Focus on specialized pro-resolving mediators and NADPH oxidase inhibitors as potential therapeutic strategies. *Pharmacol. Res.,* **162**: 105280.

Teleanu DM, Niculescu AG, Lungu, II, Radu CI, Vladacenco O, Roza E, Costachescu B, Grumezescu A M and Teleanu RI (2022). An overview of oxidative stress, neuroinflammation and neurodegenerative diseases. *Int. J. Mol. Sci.,* **23**: 5938.

Wang JL, Luo X and Liu L (2019). Targeting CARD6 attenuates spinal cord injury (SCI) in mice through inhibiting apoptosis, inflammation and oxidative stress associated ROS production. *Aging* (Albany NY), **11**: 12213-12235.

Wang M, Pan W, Xu Y, Zhang J, Wan J and Jiang H (2022). Microglia-mediated neuroinflammation: A potential target for the treatment of cardiovascular diseases. *J. Inflamm. Res.,* **15**: 3083-3094.

Wang W, You RL, Qin WJ, Hai LN, Fang MJ, Huang G H, Kang RX, Li MH, Qiao YF, Li JW and Li AP (2015). Anti-tumor activities of active ingredients in compound Kushen injection. *Acta Pharmacol. Sin.,* **36**: 676-679.

Ye D, Fairchild TJ, Vo L and Drummond PD (2022). Painful diabetic peripheral neuropathy: Role of oxidative stress and central sensitisation. *Diabet. Med.,* **39**: e14729.

Zhu T, Zhou D, Zhang Z, Long L, Liu Y, Fan Q, Chen F, Zhang X, Wu Y, Zeng H, Verkhratsky A, Zhao J and Nie H (2020). Analgesic and antipruritic effects of oxymatrine sustained-release microgel cream in a mouse model of inflammatory itch and pain. *Eur. J. Pharm. Sci.,* **141**: 105110.