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Research Article
Dantongding Formulation Alleviates Nerve Damage in Diabetic Peripheral Neuropathy Rats by Reducing Oxidative Stress Through Nrf2/Ho-1 Signaling Pathway

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Abstract

Background and Objective: Diabetic peripheral neuropathy (DPN), affecting over 50% of diabetic patients, is a major cause of ulcers and amputation, severely impacting quality of life. Despite extensive research, DPN's mechanisms remain elusive. This study aims to explore the Traditional Chinese Medicine of Dantongding, particularly through the Nrf2/HO-1-mediated oxidative stress pathway, to advance DPN treatment strategies. **Materials and Methods:** A rat model of DPN was induced using a high-fat diet and streptozotocin (STZ) comprised of six groups. Histopathological changes in the sciatic nerves of rats were observed by Hematoxylin & Eosin (H&E) and Weil staining. The changes in sciatic nerve conduction velocity were measured. Serum malondialdehyde (MDA), superoxide dismutase (SOD), Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) levels were measured. The mRNA and protein expression of Nrf2, HO-1 and p65 in the sciatic nerve was assessed using qRT-PCR and western blotting. **Results:** Compared to the control group, DPN rats exhibited characteristic changes in sciatic nerve tissue, including nerve fiber loss, vacuolar defects, axonal atrophy and myelin dissolution. Pathological damage to the sciatic nerve in the low-, medium- and high-dose dantongding formulation groups, as well as in the capsaicin group, showed varying degrees of improvement. Compared to the DPN group, the high-dose dantongding and capsaicin groups showed significant increases in sciatic nerve conduction velocity, serum SOD levels, Nrf2, HO-1 mRNA and protein levels, along with significant decreases in serum TNF-α, IL-6 and p65 mRNA and protein levels. **Conclusion:** The therapeutic effects of the Dantongding formulation on DPN in rats may be associated with the inhibition of oxidative stress, activation of the Nrf-2/HO-1 pathway and suppression of the Nuclear Factor kappa-B (NF-κB) pathway.

Key words: Diabetic peripheral neuropathy, dantongding, Nrf-2/HO-1 pathway, oxidative stress

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetic peripheral neuropathy (DPN) is a common chronic complication of diabetes with an incidence of >50%[1]. It is a major cause of ulcers and amputation in patients with diabetes and significantly affects their quality of life. Peripheral nerves are most commonly affected, involving the motor, sensory and autonomic nerves[1-3]. Symptoms include abnormal sensations such as burning, electric shock-like or cutting pain, numbness, hypersensitivity and deep muscle pain[4]. This can progress to a complete loss of sensation, leading to foot ulcers, necrosis and amputation. Pathologically, DPN is characterized by axonal atrophy, demyelination, nerve fiber loss and slowed nerve fiber regeneration[5].

Despite decades of research, the specific mechanisms underlying the development of DPN remain unclear. Current treatments primarily focus on lifestyle improvements and blood sugar control[6]. Therefore, the development of effective therapeutic strategies is crucial. The DPN is a complex process regulated by multiple factors that involve a bio-coordinated environment in which multiple factors act together[1]. Effective Traditional Chinese Medicine formulations may provide a microenvironment closer to neurophysiological needs or nerve repair and regeneration, presenting advantages over Western medicine targeting a single factor.

Dantongding (DTD), an internally and externally applied formulation used in our hospital, has demonstrated significant efficacy in DPN treatment. Formulated by multiple generations of experts, combining family experience and Yunnan minority herbal knowledge, DTD contains multitarget compounds, such as aconitine. Although its clinical efficacy has been confirmed, its exact mechanism of action in treating DPN is still under investigation.

The pathogenesis of DPN involves nerve cell damage caused by high blood sugar levels associated with metabolic pathway changes, increased oxidative stress and inflammatory reactions. Oxidative stress occurs when reactive nitrogen and oxygen free radicals exceed the capacity of the body to eliminate them, leading to the dysfunction of the body's oxidative system and tissue damage. High glucose levels result in the generation of advanced glycation end products and activation of Nuclear Factor kappa-B (NF-κB)[7,8]. Elevated pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β) due to high blood sugar contribute to nerve cell damage[7,9,10]. Addressing oxidative stress, reducing oxidative damage to nerve tissue and slowing the progressive decline in nerve function in diabetic patients are emerging strategies for preventing and treating DPN.

Furthermore, transcription factor Nrf2 plays a crucial role in the regulation of cellular oxidative stress. Activation of the Nrf2-ARE signaling pathway induces detoxification enzymes, providing protection against oxidative stress. Recent studies have suggested that various natural compounds can activate Nrf2, offering antioxidative and anti-inflammatory effects[7,11,12]. External activation of the Nrf2 pathway through natural products seems to support the endogenous defense system, providing a feasible approach for preventing and treating diabetic peripheral neuropathy.

This study aimed to elucidate the specific mechanisms through which Illusion Pain Tincture improves diabetic peripheral neuropathy by regulating the Nrf2-mediated oxidative stress pathway. Animal experiments will be conducted to provide a theoretical foundation for the continued development and application of Traditional Chinese Medicine for the treatment of DPN.

MATERIALS AND METHODS

Study area: The study was carried out at the Yunnan University of Traditional Chinese Medicine, located in Kunming, China. The research spanned a period from October, 2021 to September, 2023.

Experimental animals: Sixty specific pathogen-free (SPF) male Wistar rats, aged 4-5 weeks and weighing 100-120 g, were procured from the Animal Experiment Center of Kunming Medical University and met the criteria for animal quarantine. The conditions were maintained as pathogen-free, with a room temperature of 22-24 °C, a relative humidity of 45 to 65% and a light-dark cycle (on at 8 am and off at 8 pm).

Ten rats were randomly assigned to the blank control group and fed a normal diet, while the remaining rats were fed a high-fat diet. After 4 weeks of feeding, the rats were fasted overnight, followed by intraperitoneal injection of streptozotocin (STZ) (35 mg kg^{-1}) the next day to establish a diabetic peripheral neuropathy (DPN) model. Changes in blood glucose levels and pain behaviors were monitored weekly. Rats with blood glucose levels >16.7 mmol L^{-1} from 3 days post-STZ injection and persistent neuropathic pain (tactile pain threshold $\leq$7.5 g and thermal pain threshold $\leq$10 s) after 4 weeks were selected as the DPN neuropathic pain animal model. They were then randomly divided into six groups: Model, capsaicin and high-, medium- and low-dose DTD.

Grouping of animals, dosage and administration method: Prior to the experiment, the hind limbs of the rats were depilated using a depilating agent. After 24 hrs, rats without skin damage were selected for skin administration and the cream was evenly applied to both hind limbs. The

Table1: Primer sequence for real-time PCR

Gene	Forward primer (5'-3')	Reverse primer(5'-3')
NRF2	AGGTTGCCCACATTCCCAAA	AGGGCAAGCGACTGAAATGT
p65	TGAACTCTGCCCAGACCGAT	GTCTGGATGCGCTGGCTAAT
HO-1	GCGAAACAAGCAGAACCCAG	TACGTAGTGCTGTGTGGCTG
GAPDH	CAGTGCCAGCCTCGTCTCAT	AGGGGCATCCACAGTCTTC

high-, medium- and low-dose DTD groups received three doses of the raw drug (3.0, 1.5 and 0.75 g mL^{-1}, respectively), administered twice daily in the morning and evening. The capsaicin ointment group received topical administration of capsaicin ointment (0.025%) twice a day. The normal and model groups were treated with blank cream (made of various matrices in regenerated pain tincture cream) for 12 weeks. Except for the blank group, the rats in all other groups were fed a high-fat diet. The experimental drugs were sourced from the pharmacy of the Yunnan Traditional Chinese Medicine Hospital.

Sciatic nerve conduction velocity measurements in rats: The BL-420 S biological function transducer system (Chengdu Taimee Technology Co. Ltd., Chengdu, China) was used to assess the right sciatic nerve of rats by measuring both Motor Nerve Conduction Velocity (MNCV) and Sensory Nerve Conduction Velocity (SNCV). For MNCV measurements, the stimulating electrode (Channel 1) was positioned at the proximal end of the nerve, whereas the recording electrode (Channel 2) was placed at the distal end to capture the evoked action potential. The MNCV was calculated using the formula:

$$MNCV = \frac{\text{Length between the stimulating electrodes of channels 1 and 2 (min)}}{\text{Time difference between the peak of the action potential in channels 1 and 2 (sec)}}$$

For SNCV measurements, the positions of the stimulating and recording electrodes were swapped with the recording electrode of Channel 2 located at the proximal end of the nerve. The SNCV was calculated using the following formula:

$$SNCV = \frac{\text{Length between the recording electrode of channels 1 and 2 (min)}}{\text{Time difference between the action potential peaks of channels 1 and 2 (sec)}}$$

Morphological observation of sciatic nerve: After completing the 12-week treatment period, the right sciatic nerves of the rats were extracted, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 μm. These sections were stained with H&E and Weal's myelin sheath stain, enabling the microscopic observation of morphological changes in the sciatic nerves.

Expression of oxidative stress factors and inflammatory factors in rat serum: Thirty minutes after the final administration, ten blood samples of each group were collected from the inner canthus vein of rats to obtain serum for the determination of malondialdehyde (MDA), superoxide dismutase (SOD), Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) levels following the manufacturer's instructions. All kits were sourced from Nanjing Jiancheng BioEngineering Institute (Nanjing, China).

Quantitative real-time reverse transcription Polymerase Chain Reaction (qPCR): Total RNA was extracted using TRIzol reagent and mRNA was isolated using an RNA Ultrapure Extraction Kit (CWBio). The RNA concentration and purity (OD260/OD280) were assessed using a UV-visible spectrophotometer (Nanodropone, Thermo Scientific, USA) and cDNA synthesis was performed using an RNA Reverse Transcription Kit (CWBio). Quantitative real-time PCR was performed using a fluorescent PCR apparatus (CFX Connect™ Real-Time, Bio-rad Life Medicine, USA). Relative gene expression was calculated using the $2^{-\Delta\Delta ct}$ method, with GADPH as an internal control. The primer sequences used were listed in Table 1.

Western blotting: Rat sciatic nerve tissue was collected and RIPA lysate was added and ground with a tissue grinder to extract the total tissue protein. Following centrifugation for 10 min, the supernatant was collected and total protein was quantified using the Pierce™ BCA Protein Quantification Kit (Thermo Scientific). After denaturation, the protein samples were subjected to Sodium Dodecyl Sulfate Gel Electrophoresis (SDS-PAGE) for 1.5 hrs. Subsequently, the membrane was rotated at 300 mA for 1 hr. The PVDF membranes (Millipore) were blocked with skim milk powder and primary antibodies Nrf2, HO-1 and NF-κB (p65) were detected overnight at 4 . The next day, the PVDF membrane was incubated with the PVDF membrane for 2 hrs at room temperature. Subsequently, the membranes were imaged and analyzed.

Ethical consideration

Ethical approval and consent to participate: The experimental protocols conducted in this article with animals were approved by the Ethical Committee of Yunnan University of Traditional Chinese Medicine (No. R-062022G010). All the participants provided written informed consent. All methods were carried out in accordance with the relevant guidelines and regulations.

Statistical analysis: Data analysis was performed using SPSS 20.0 and GraphPad Prism 8.0. All data are expressed as Mean±Standard Deviation (SD). Unpaired Student's t-test and One-way Analysis of Variance (ANOVA) were used for statistical comparisons between two and multiple groups, respectively. Statistical significance was set at $p<0.05$.

RESULTS

Pathological examination and related behavioral experiments indicate that DTD can alter nerve damage in rats with diabetic peripheral neuropathy: The results of H&E staining (Fig. 1a) showed that the nerve fibers in the normal group had a large diameter, dense arrangement and uniform thickness of the myelin sheath wrapped around the axons of the medullary nerve fibers. In the DPN group, nerve fibers were of varying sizes, cells exhibited vacuolar defects and axons were atrophic. The morphology of cells in the capsaicin and high-dose DTD groups was similar to that of the control group, with no vacuolated cells observed. Compared with the model group, the medium-dose DTD group showed some improvement, with a decrease in the number of vacuolated defect cells, whereas the low-dose DTD group exhibited a morphology similar to that of the DPN group.

Weil's staining (Fig. 1a) showed no reduction in sheath cells in the sciatic nerve sheath of normal rats and no myelinolysis or demyelination was observed. The DPN group showed a greater degree of myelinolysis and demyelination and a decreased number of sheath cells. There was no reduction in sheath cells in the sciatic nerve sheath and no myelinolysis in rats treated with capsaicin or high-dose DTD. Mild myelinolysis and demyelination were observed in the sciatic nerves of rats in the medium- and low-dose DTD

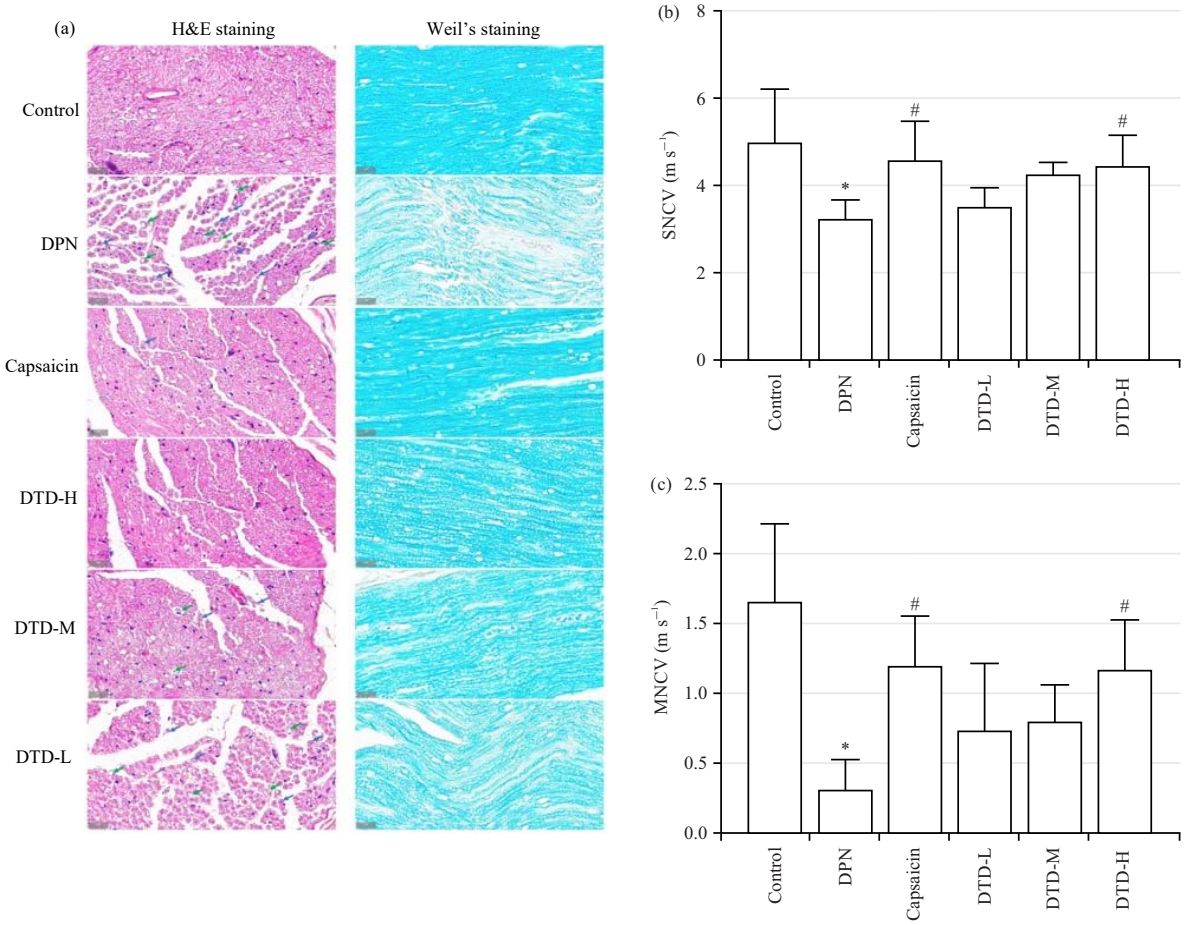

Fig. 1(a-c): DTD can effectively improve the pathological and behavioral responses in rats with diabetic peripheral neuropathy, (a) Effects of DDT on pathological changes and nerve conduction velocity of sciatic nerve in DPN rats, (b) Sensory nerve conduction velocity, SNCV and (c) Motornerve conduction velocity, MNCV

Histopathology changes of sciatic nerve in rats (HE, 80×), vacuolar-like defects, demyelination changes and axonal shrinkage, histopathology changes in sciatic nerve in rats (Weil's myelin staining, 80×), data was presented as the Mean±SD (n = 6), *$p<0.05$ vs control group and #$p<0.05$ vs DPN group

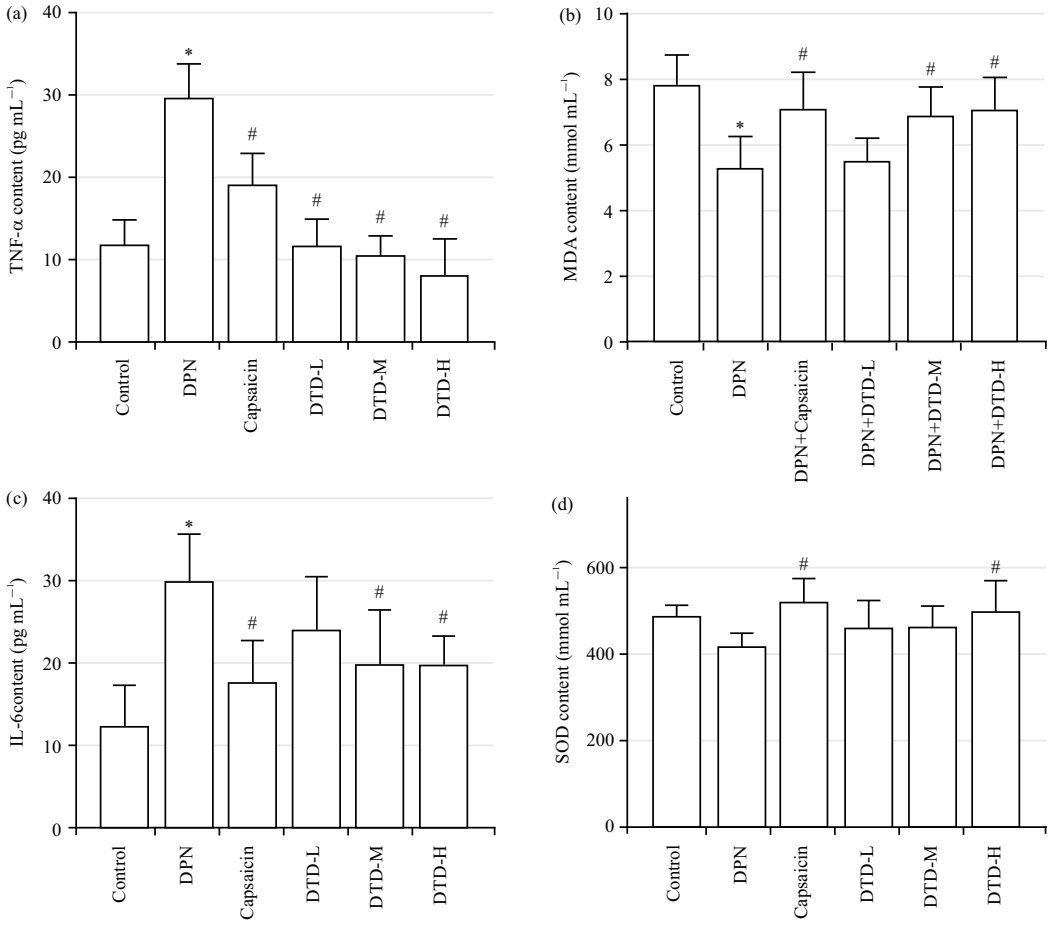

Fig. 2(a-d): Effects of DDT on serum oxidative stress and expression of inflammatory factors in DPN rats, (a) MDA, (b) SOD, (c) TNF-α and (d) IL-6

Data was presented as the Mean±SD (n = 6), *p<0.05 vs control group and #p<0.05 vs DPN group

groups, respectively. These results suggest that DTD intervention can alleviate pathological damage to sciatic nerve tissue in DPN rats.

Furthermore, we measured the motor and sensory nerve conduction velocities of the sciatic nerve to determine whether nerve damage was present in DPN rats and whether DTD could alleviate nerve damage. The motor and sensory nerve conduction velocities of DPN rats were significantly lower than those of control rats (p<0.05, Fig. 1b-c), indicating that DPN is a nerve injury. No significant differences were observed between the capsaicin and DTD groups. However, the motor and sensory nerve conduction velocities of the capsaicin and DTD groups were higher than those of the control rats, indicating that DTD can improve nerve damage in DPN rats.

DTD can effectively alleviate oxidative stress and inflammatory factors in rats with diabetic peripheral neuropathy: To assess the effect of DTD on oxidative stress and inflammation in rats with diabetic peripheral neuropathy (DPN), serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) were determined using relevant kits. Compared to the control group, the model group exhibited significantly elevated levels of MDA, TNF-α and IL-6 (p<0.05, Fig. 2). Conversely, the capsaicin and DTD groups showed significantly reduced levels of MDA, TNF-α and IL-6 compared to those in the model group (p<0.05). These findings indicate that DTD mitigates oxidative stress and inflammatory damage in rats with diabetic peripheral neuropathy.

DTD exerts anti-inflammatory effects in treating diabetic peripheral neuropathy through the Nrf2/HO-1/NF-κB molecular signaling pathway: To elucidate the mechanism by which DTD ameliorates diabetic peripheral neuropathy by modulating Nrf2 and related mediators, gene and protein expression levels of Nrf2, HO-1 and p65 (NF-κB) were assessed by qPCR and WB (Fig. 3 and 4). Relative to the control group,

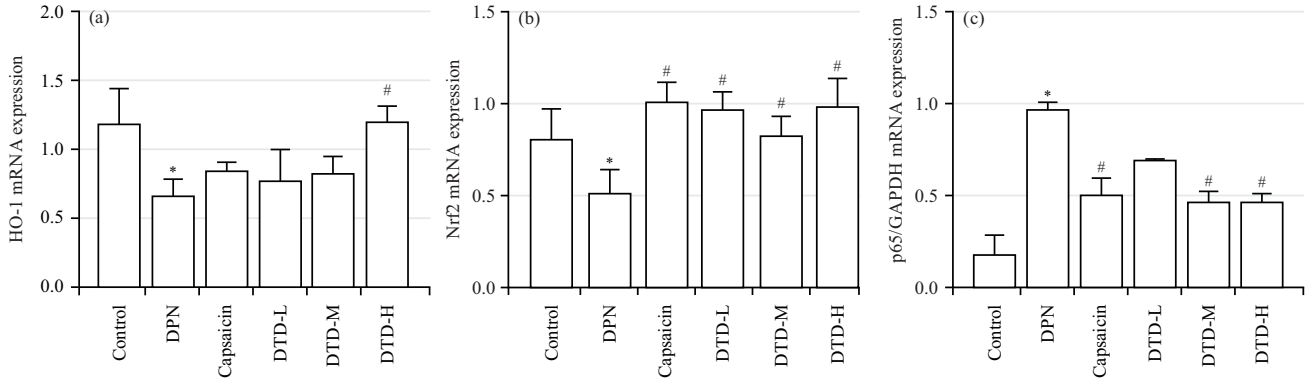

Fig. 3(a-c): Effect of DDT on Nrf2/HO-1/NF-κB pathway in DPN rats, mRNA expression of (a) HO-1, (b) Nrf2 and (c) p65
Data was presented as the Mean±SD (n = 5) *p<0.05 vs control group and #p<0.05 vs DPN group

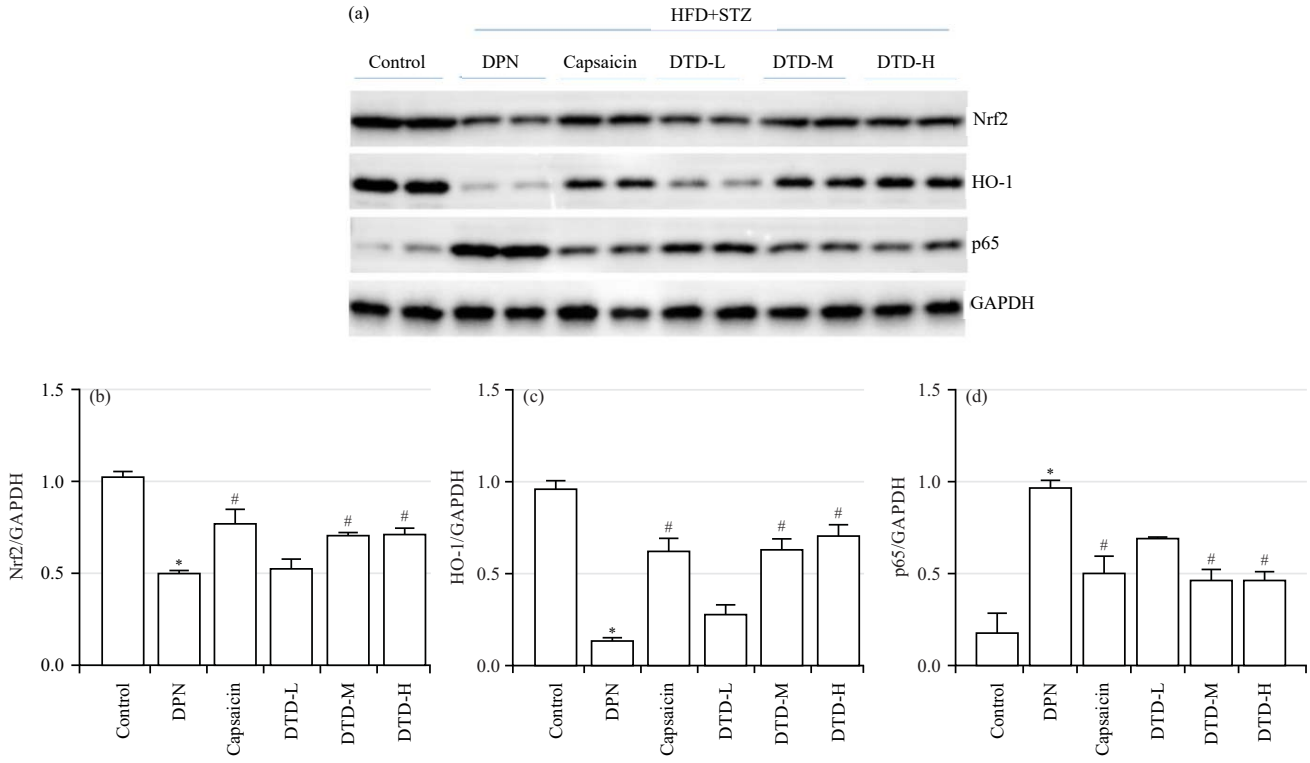

Fig. 4(a-d): Effect of DDT on Nrf2/HO-1/NF-κB pathway in DPN rats, (a) Representative western blot images and (b) Densitometric analysis of Nrf2/GADPH, (c) HO-1/GADPH and (d) p65/GADPH, respectively
Data was presented as the Mean±SD (n = 3), *p<0.05 vs control group and #p<0.05 vs DPN group

both the mRNA and protein levels of Nrf2 and HO-1 in the DPN group were significantly downregulated, whereas the mRNA and protein levels of NF-κB were markedly upregulated. Compared with the DPN group, the high-dose DTD group demonstrated significantly upregulated mRNA and protein levels of Nrf2 and HO-1, along with significantly downregulated mRNA and protein levels of p65. These results indicate that DTD alleviates diabetic peripheral neuropathy damage in rats by activating the Nrf2/HO-1 pathway and inhibiting the NF-κB pathway.

DISCUSSION

The study presents compelling evidence on the effectiveness of dantongding (DTD) in managing diabetic peripheral neuropathy (DPN). The DTD, with its two decades

of clinical use, appears to notably improve microcirculation and nerve conduction velocity in DPN, addressing both the symptoms and potential complications of the condition. The DTD treatment results in reduced blood glucose levels, increased body mass and significant improvement in the structural integrity of the sciatic nerve in DPN rats. These improvements include the mitigation of nerve fiber loss, vacuolar defects, axon atrophy, myelinolysis and demyelination.

Additionally, the therapeutic effects of DTD extend to reducing mechanical and heat sensitization, accelerating motor and sensory nerve conduction velocities and alleviating liver and kidney function injuries in DPN rats. This suggests a broader systemic benefit beyond the direct impact on neuropathy.

The underlying mechanisms of DTD's action were explored through the Nrf2/HO-1 and NF-κB pathways. The Nrf2/HO-1 pathway is crucial for detoxifying reactive free radicals, while the NF-κB pathway is involved in inflammatory responses. The DTD's ability to reduce oxidative stress and inflammation is evidenced by decreased levels of MDA, TNF-α and IL-6. The inhibition of NF-κB and increased expression of Nrf2 and HO-1 indicate DTD's potential in activating the Nrf2/HO-1 pathway and inhibiting the NF-κB pathway.

Diabetic peripheral neuropathy (DPN) is a prevalent cause of chronic pain syndromes and a significant risk factor for complications such as foot ulcers and lower limb amputation[11,13]. Approaches to this condition primarily involve blood sugar control, along with the use of antioxidants and neurotrophic drugs to enhance microcirculation. However, the drawbacks of Western medicine, including side effects, long-term drug usage-related economic burden and drug resistance, necessitate alternative therapeutic options. The pathogenesis of DPN remains unclear, although chronic hyperglycemia-induced oxidative stress is widely considered to be a pivotal factor in its development. Recent studies have indicated a close association between oxidative damage induced by oxidative stress and the apoptotic pathway in the pathogenesis of DPN[14-16].

The Nrf2/HO-1 pathway, essential for detoxifying reactive free radicals and the NF-κB pathway, implicated in inflammatory injury, were targeted to explore the mechanisms underlying DTD's effects[17,18]. The DTD reduces oxidative stress and inflammation by inhibiting MDA, TNF-α and IL-6 levels in DPN rats. Furthermore, DTD inhibited NF-κB and increased Nrf2 and HO-1 expression, suggesting its ability to activate the Nrf2/HO-1 pathway and inhibit the NF-κB pathway.

The DTD emerges as a promising alternative therapeutic approach for DPN, addressing the limitations of conventional Western medicine in treating this condition. Its dual action of improving nerve health and systemic factors, coupled with a mechanism that involves modulation of oxidative stress and inflammation, underscores its potential as a comprehensive treatment strategy for DPN.

CONCLUSION

The DTD demonstrated therapeutic potential in ameliorating hyperglycemia-induced DPN. Through the activation of the Nrf2/HO-1 pathway and inhibition of the NF-κB pathway, DTD effectively reduced oxidative stress and inflammation in DPN rats. Its anti-inflammatory and analgesic effects contribute to the alleviation of nerve injury in rats with diabetic peripheral neuropathy. These findings provide novel insights into the treatment of DPN using DTD and shed light on its potential therapeutic mechanisms.

SIGNIFICANCE STATEMENT

Dantongding formulation shows neuroprotective effects on diabetic peripheral neuropathy (DPN) in rats, potentially through the Nrf2/HO-1 pathway. In this study, a rat DPN model was created using a high-fat diet and streptozotocin. Groups included a control, a model group, a capsaicin group and three DTD dosage groups. We assessed sciatic nerve damage, nerve conduction and serum levels of malondialdehyde, superoxide dismutase, TNF-α and IL-6. Also, Nrf2, HO-1 and p65 expression in sciatic nerves were measured. The DTD significantly improved sciatic nerve damage and conduction, increased Nrf2 and HO-1 and decreased TNF-α, IL-6 and p65. These results suggest DTD's efficacy in DPN is linked to reducing oxidative stress, activating Nrf-2/HO-1 and inhibiting NF-κB pathways.

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