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# Quercetin Alleviates Diabetic Peripheral Neuropathy by Regulating Axon Guidance Factors and Inhibiting the Rho/ROCK Pathway in vivo and in vitro

Wei Song[1,2,*], Yaoyang Li[1,*], Yifan Jia[1], Lingling Xu[3], Lin Kang[4], Yunshuang Yang[5], Shuyu Wang[1], Qian Zhang[1], Qunli Wu[1]

[1]Department of Traditional Chinese Medicine, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, 100730, People's Republic of China; [2]Institute of Clinical Medicine, National Infrastructures for Translational Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing, 100730, People's Republic of China; [3]Department of Endocrinology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, 100730, People's Republic of China; [4]Department of Geriatric, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, 100730, People's Republic of China; [5]Department of Preventive Medicine, Beijing Longfu Hospital, Beijing, 100010, People's Republic of China

*These authors contributed equally to this work

Correspondence: Qian Zhang; Qunli Wu, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100730, People's Republic of China, Email zhangqian1@pumch.cn; chinlie@163.com

**Purpose:** The axon guidance factors and Rho/ROCK pathway play crucial roles in axon protection and nerve repair and has been implicated in the development of diabetic peripheral neuropathy (DPN). This study investigates the protective effects of quercetin against DPN, focusing on axon guidance factors and Rho/ROCK pathway.

**Methods:** DPN was induced by intraperitoneal injection of streptozotocin (STZ) to Sprague-Dawley rats. The DPN model rats were allocated into three groups and administered quercetin at two different doses (30 mg/kg/day and 60 mg/kg/day) or a placebo. Concurrently, healthy rats were divided into two groups and administered either a placebo or quercetin (60 mg/kg/day). Administration was initiated 8 weeks post-STZ injection and continued for a duration of six weeks. To assess quercetin's neuroprotective effects, biochemical analyses, neurological function tests (mechanical threshold, thermal response latency, motor nerve conduction velocity), and morphological assessments via transmission electron microscopy were conducted. Immunofluorescence and immunohistochemical assays were performed on sciatic nerve tissue and high glucose-induced RSC96 rat Schwann cells to explore quercetin's pharmacological effects on DPN.

**Results:** Quercetin exhibited neuroprotective effects on both DPN rats and RSC96 cells exposed to high-glucose. A six-week administration of quercetin at both doses significantly improved the peripheral neurological functions and alleviated the pathological changes in sciatic nerve of DPN rats ($P<0.05$). Mechanistically, quercetin markedly upregulated the expressions of axonal growth factors, Slit-2 and Netrin-1 in vivo and in vitro ($P<0.05$), while inhibiting the aberrant activation of Rho/ROCK signaling pathway in the sciatic nerve of DPN rats.

**Conclusion:** Our findings suggest that quercetin improves DPN through a novel mechanism, indicating its potential as a therapeutic agent for DPN therapy.

**Keywords:** quercetin, diabetic peripheral neuropathy, Rho/ROCK pathway, axon guidance factor, Netrin-1, Slit-2

## Introduction

Diabetic peripheral neuropathy (DPN), a prevalent and severe complication of diabetes, affects approximately 50% of individuals with type 1 or type 2 diabetes.[1] Symptoms of DPN, such as numbness, pain, paresthesia, weakness, and balance issues, significantly impact patients' quality of life and contribute to escalating healthcare costs.[2] The

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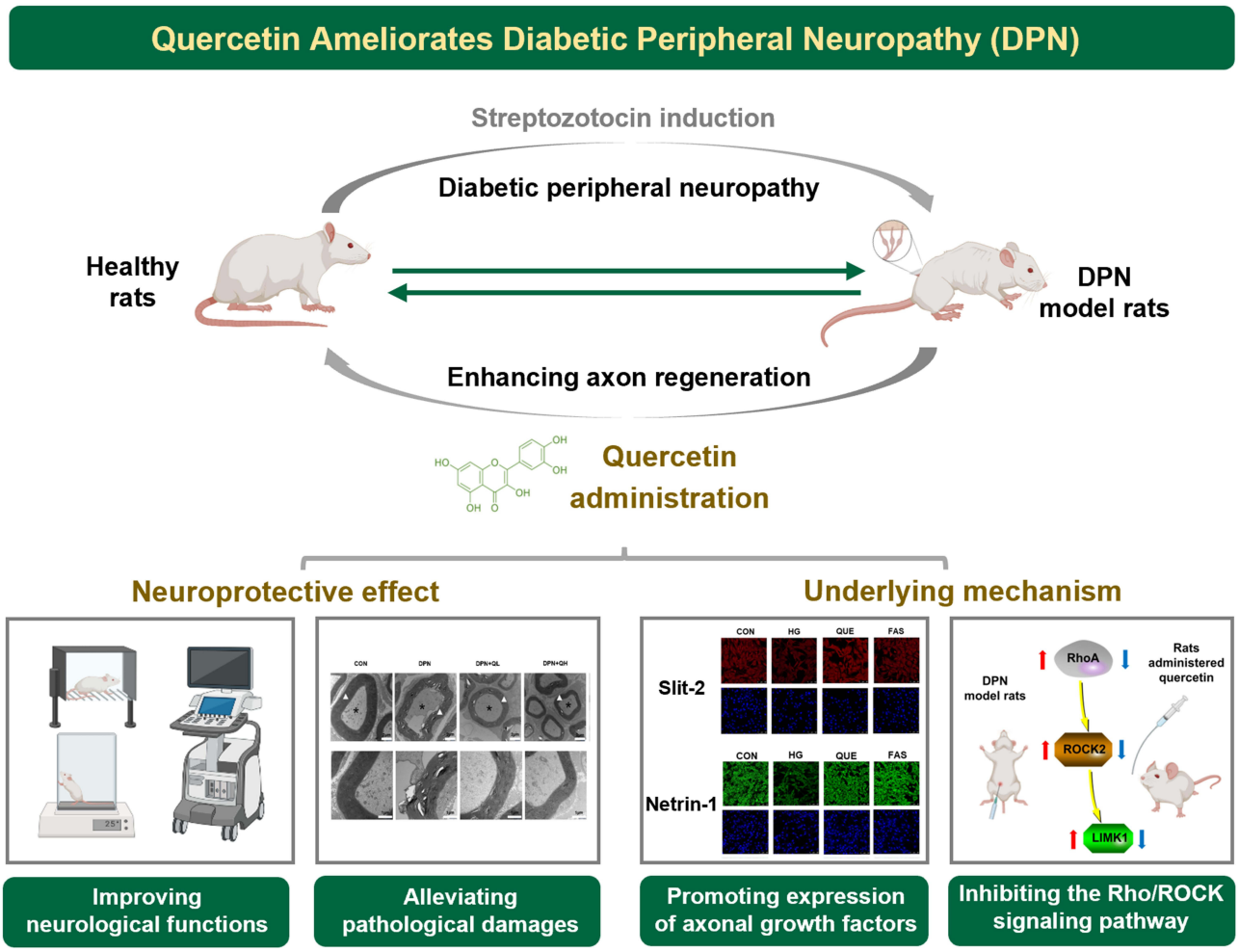
**Graphical Abstract**




pathogenesis of DPN is closely linked to impaired peripheral nerve regeneration and repair in hyperglycemic conditions. The myelin sheath surrounds axons in the peripheral nervous system, and DPN is characterized by the myelin breakdown and axonal shrinkage.[3] During the regeneration of peripheral nerves, axons extend along specific routes towards their target sites, with precise targeting and connection formation being crucial stages in nerve repair.[4]

Axon guidance factors are secreted proteins that play essential roles in either attracting or repelling axons, thereby modulating the rearrangement of the actin cytoskeleton within the growth cone, a specialized structure located at the tip of axons.[5] Among these factors, the Netrins and Slits families are notable, with Netrin-1 binding to DCC and UNC5A receptors on the growth cone to guide axon growth direction.[6] Slit guidance ligand 2 (Slit-2), as a member of the Slits protein family, interacts with the receptor Robo and is involved in neuronal migration, axonal growth, and collateral formation during nerve repair.[7]

Rho family proteins are key regulators of the actin cytoskeleton, influencing cellular morphology and motility by regulating cytoskeletal reorganization through their downstream effector, Rho kinase (ROCK).[8] Rho proteins and their associated signaling molecules are critical to axon-directed signaling pathways due to their regulation of the cytoskeleton.[9] The activation of RhoA and ROCK has been demonstrated in the sciatic nerve of diabetic rats induced by streptozotocin (STZ) and a high-fat diet.[10] ROCK inhibitors, such as fasudil, Y-27632, and H1152, have demonstrated

potential in the treatment of diabetic neuropathy.[11] The inhibition of Rho/ROCK pathway also promotes axon regeneration and nerve repair following peripheral nerve injury.[12]

Quercetin is a natural flavonoid found ubiquitously in a variety of vegetables, fruits, and medicinal plants.[13,14] It exhibits potent bioactive effects, including antioxidation, anti-inflammation, and metabolic regulation, thereby providing neuroprotective effects.[15] It has been reported that quercetin could alter the cytoskeleton to inhibit abnormal cell migration, as well as suppressing Rho activation in other disease models.[16–18] Quercetin has been found to ameliorate neurological damage, such as promoting nerve regeneration after sciatic nerve-crush injury,[19] inhibiting neuronal apoptosis after cerebral ischemia/reperfusion injury,[20] and protecting neurons and Schwann cells from high-glucose-induced injury.[21–23] Previous studies also revealed that quercetin shows potential in DPN treatment by alleviating neuropathic pain and improving nerve conduction velocity of STZ-induced rats.[23,24] Our previous study have shown quercetin upregulated nerve regeneration-associated proteins such as myelin basic protein and myelin protein zero in the peripheral nerve of STZ-induced diabetic rats.[23] Therefore, we assumed that quercetin might protect the axon of DPN rats by inhibiting the Rho/ROCK signaling pathway. The effects of quercetin on axon guidance factors and Rho/ROCK pathway, are yet to be elucidated. Schwann cells, the myelinating glia of the peripheral nervous system not only form myelin but also provide trophic support to neurons and axons. Therefore, following the animal experiments, the present study employed a high-glucose-induced Schwann cell model for further validation.

This study investigates the neuroprotective effect of quercetin on a DPN rat model, and further explores the underlying mechanisms, specifically focusing on axon guidance factors and the Rho/ROCK signaling pathway in vivo and in vitro.

# Materials and Methods

## Experimental Animals

Healthy male Sprague-Dawley (SD) rats weighing 200–220 g were obtained from Vital River Laboratory Animal Technology (Beijing, China). The rats were housed in a specific pathogen-free environment, maintained at a temperature of 21 ± 1 °C and under a 12-h:12-h light: dark cycle. All rats were acclimated for three days before the study, and were provided with ordinary chow and water ad libitum. The animal experiment was conducted following the Guidelines of the Care and Use of Laboratory Animals is-sued by the Chinese Council on Animal Research. All procedures were approved by the Institutional Animal Care and Use Committee of the Peking Union Medical College Hospital (Approval No: XHDW-2021-00).

## Grouping and Treatment

After overnight fasting, the diabetic rat model was established by a single intraperitoneal injection of 60 mg/kg STZ (Sigma, USA). After 72 h of STZ injection, blood glucose levels in tail vein were measured using Accu-Chek glucose meter (Roche, Ireland). If the blood glucose concentration was ≥ 16.7 mmol/L on two different days, the diabetic rat model was considered to be successfully established. Then the diabetic rats were randomly divided into three groups (n = 5 per group): diabetes model group (DPN), low-dose quercetin treatment group (DPN + QL), high-dose quercetin treatment group (DPN + QH). At the same time, age-matched healthy rats were used as controls and divided in to two groups (n = 5 per group): normal control group (CON), and healthy rats with high-dose quercetin treatment group (CON + QH). Eight weeks after STZ injection, the rats in DPN + QL group were administered quercetin intragastrically at a dose of 30 mg/kg body weight daily, the rats in DPN + QH and CON + QH groups were intragastrically administered with quercetin at 60 mg/kg body weight daily, and the CON and DPN groups were received an equivalent volume of distilled water. The intervention lasted six weeks.

Blood glucose levels and body weight were monitored every four weeks over the course of the experiment. Following the treatment, all rats were anesthetized deeply through intraperitoneal injection of 10% chloral hydrate (3 mL/kg). Motor nerve conduction velocity was initially assessed, followed by the collection of blood from the carotid artery prior to sacrifice. Blood was centrifuged at 3000 rpm for 15 min, and the resulting serum samples were stored at −80 °C. The

sciatic nerves were harvested, with one segment fixed in 10% phosphate-buffered formalin for immunofluorescence and immunohistochemical analysis, and the other segment fixed in 2.5% glutaraldehyde for electron microscopy.

## Mechanical Threshold and Thermal Response Latency

The mechanical pain threshold of each group of rats was determined using the Von Frey pain meter (IITC Life Science, USA), following a previously described method.[25] The transparent glass enclosure was positioned on a wire mesh platform at a height of 50 cm above the experimental surface, permitting complete exposure of the hind paw when each rat was placed inside. Von Frey filaments were applied vertically to stimulate the plantar surface of the hind paw, with the sudden withdrawal of the paw being considered a positive response and the corresponding threshold value recorded. Each rat underwent three mechanical pain evaluations, separated by 5-min intervals.

The tail flick latency test was used to assess the tail flick latency in rats. Rats were restrained with a retainer to expose the tail, and a mark was placed 5 cm from the tail tip. Subsequently, the tail was immersed in water maintained at 50 °C, with the tail tip submerged up to the marked point. The time taken for the tail to flick was recorded using a stopwatch, to determine the tail flick latency. The rat tail was briefly immersed for no longer than 15 seconds to prevent tissue damage. Measurements were taken at 5-min intervals, three times consecutively.

## Motor Nerve Conduction Velocity

Motor nerve conduction velocity (MNCV) was measured using the EMG/EP system (NCC Medical Co. Shanghai, China), following a previously established method.[25] The rats were first anesthetized, and their body temperature was maintained between 35 and 37 °C using a heat lamp. A pair of stimulating electrodes was positioned at the sciatic notch of the sciatic nerve (A), and the recording electrodes were positioned in the muscle belly of the gastrocnemius muscle (B). A ground electrode was pierced at the root of the rat's tail. The latency (t1) was recorded when the rat was stimulated by a single pulse. Then the recording electrodes were repositioned at the tibial notch of the tibial nerve (C), and the latency (t2) was recorded when the rat was stimulated by a single pulse. The distance between B and C was recorded as d. Finally, MNCV was calculated by the equation: $\mathrm{MNCV(m/s)} = \mathrm{d}/(\mathrm{t2} - \mathrm{t1})$.

## Biochemical Examinations

The serum levels of alanine aminotransferase (ALT), albumin (ALB), aspartate aminotransferase (AST), and creatinine (CREA) were performed on a fully automatic biochemical analyzer (7180, Hitachi, Japan). The serum levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), and endothelial nitric oxide synthetase (eNOS), were determined by Rat IL-6 ELISA Kit (Abcam, Cat. No. ab234570), Rat IL-1β ELISA Kit (Abcam, Cat. No. ab255730), and Rat eNOS ELISA Kit (mlBio, Cat. No. mlsw_E2054), respectively. The kits were utilized as per manufacturer's instructions. Relative absorbance was determined by a microplate reader (BIO-RAD microplate reader-550).

## Transmission Electron Microscopy (TEM)

Ultrastructural changes in the sciatic nerves were assessed with a transmission electron microscope (JEOL, Japan). The sciatic nerves of rats, fixed with 2.5% glutaraldehyde, were rinsed three times with PBS and dehydrated in a series of alcohol and acetone gradients. Subsequently, the samples were embedded in epoxy resin. Thin sections measuring 1 μm thickness were cut using a diamond knife, stained with toluidine blue, and examined under light microscopy. Following the cutting of the block into 70 nm slices, the specimens were stained with uranyl acetate (20 min) and lead citrate (15 min). Finally, the ultrastructure of sciatic nerve was examined and captured photographically.

## Immunofluorescence and Immunohistochemical Assay

The sciatic nerves were fixed, embedded in paraffin wax, and sliced into 4-μm sections before being placed on a polylysine-coated slide. Following dewaxing and gradient alcohol dehydration, antigen retrieval was conducted and endogenous peroxidase activity was blocked with a 3% H2O2 solution. The sections were then treated with a blocking solution for 20 min and incubated overnight with Slit-2 antibodies (1:100, Abcam, Cat. No. AB7665), followed by washing and subsequent incubation with a secondary antibody (1:200, proteintech, Cat. No. RGAR004) at room

temperature for 2 h. DAPI and an anti-quenching agent were applied before sealing the glass cover. The resulting image was captured using a laser scanning confocal microscope (Nikon, Japan).

The fixed sciatic nerves were embedded in paraffin wax, cut into 4-μm thick slices, and placed on a polylysine-coated slide. After dewaxing, gradient alcohol dehydration, and antigen repair, endogenous peroxidase activity was blocked with 3% H2O2 solution. The sections were then treated with a blocking solution for 20 min, and the primary antibodies RhoA (1:1000, Abcam, Cat. No. ab187026), ROCK2 (1:200, ABclonal, Cat. No. A5698), and LIMK1 (1:200, ABclonal, Cat. No. A23948) were applied overnight at 4 °C. After washing with PBS three times (5 min each time), the secondary antibody (ZSGB-BIO, Cat. No. PV-9001) was added and incubated at room temperature for 1 h. After DAB color development, hematoxylin was restained, dehydrated and sealed. Images were taken by a biopsy scanner (3D histech) and positive images were analyzed using Image-J version 1.46.

## Cell Experiment

### Cell Culture and Grouping

Rat Schwann cell line (RSC96) was purchased from Cell Resource Center (IBMS, CAMS/PUMC, Peking, China). RSC96 cells were cultured in a medium with 10% fetal bovine serum in an atmosphere of 5% CO2 and 95% air at 37 °C. The RSC96 cells were categorized into four experimental groups: the normal glucose group (CON), high glucose group (HG), quercetin-treated high glucose group (QUE), fasudil group (FAS). The CON group was incubated in a normal glucose concentration at 25 mmol/L, while the other groups were incubated in a high glucose concentration at 50 mmol/L (Supplementary Figure S1). The CON group and HG group were treated with dimethyl sulfoxide (DMSO) for 72 h, while the QUE and FAS groups were treated with quercetin (10 μM, in DMSO) and fasudil (40 μM, in DMSO), respectively, for 72 h (Supplementary Figures S2 and S3).

### Immunofluorescence

RSC96 cells were utilized, with a cell concentration of $5\times10^4$/mL. A cell suspension was prepared using the designated medium for each group. The cells were then inoculated into 24-well plates coated with polylysine slides at a volume of 250 μL per well and incubated for 72 h. Subsequently, the cells were fixed with 4% paraformaldehyde overnight at 4 °C and washed three times with PBS for 5 min each time. A 1% TritonX-100 solution prepared with PBS was applied for 15 min, followed by a wash with PBS. Subsequently, a 3% H2O2 solution was applied at room temperature for 10 min to eliminate endogenous peroxidase, followed by another wash with PBS. A 5% BSA solution was then applied and sealed for 30 min and then incubated with primary antibodies: NTN1 (1:1000, Thermo Fisher Scientific, Cat. No. PA5-95725), DCC (1:200, Santa Cruz, Cat. No. sc-515834), UNC5A (1:500, Affbiotech, Cat. No. DF9679), Slit-2 (1:200, Santa Cruz, Cat. No. sc-514499), and ROBO1 (1:100, Proteintech, Cat. No. 20219-1-AP) at 4°C overnight. The samples were washed three times with PBS for 5 min each time, followed by the addition of a second antibody diluent (prepared at a 1:100 ratio) and incubation at 37 °C for 2 h. The samples were then washed three times with PBS for 5 min each time, stained with DAPI for 10 min, and washed with PBS three additional times. The images were examined using a fluorescence microscope and subsequently captured. The fluorescence intensity of the images was analyzed utilizing Image J image analysis software.

## Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics 26 (US) and GraphPad Prism 9 (US). Data were presented as group mean ± standard deviation (SD). Group comparisons were made using one-way analysis of variance (ANOVA) followed by post hoc LSD tests. Statistical significance was established at $p < 0.05$.

# Results

## Animal Experiments

Our previous study demonstrated that diabetic rats developed peripheral neuropathy eight weeks after STZ injection.[23] Subsequently, quercetin treatment was initiated at this time point and administered for six weeks (Figure 1A).

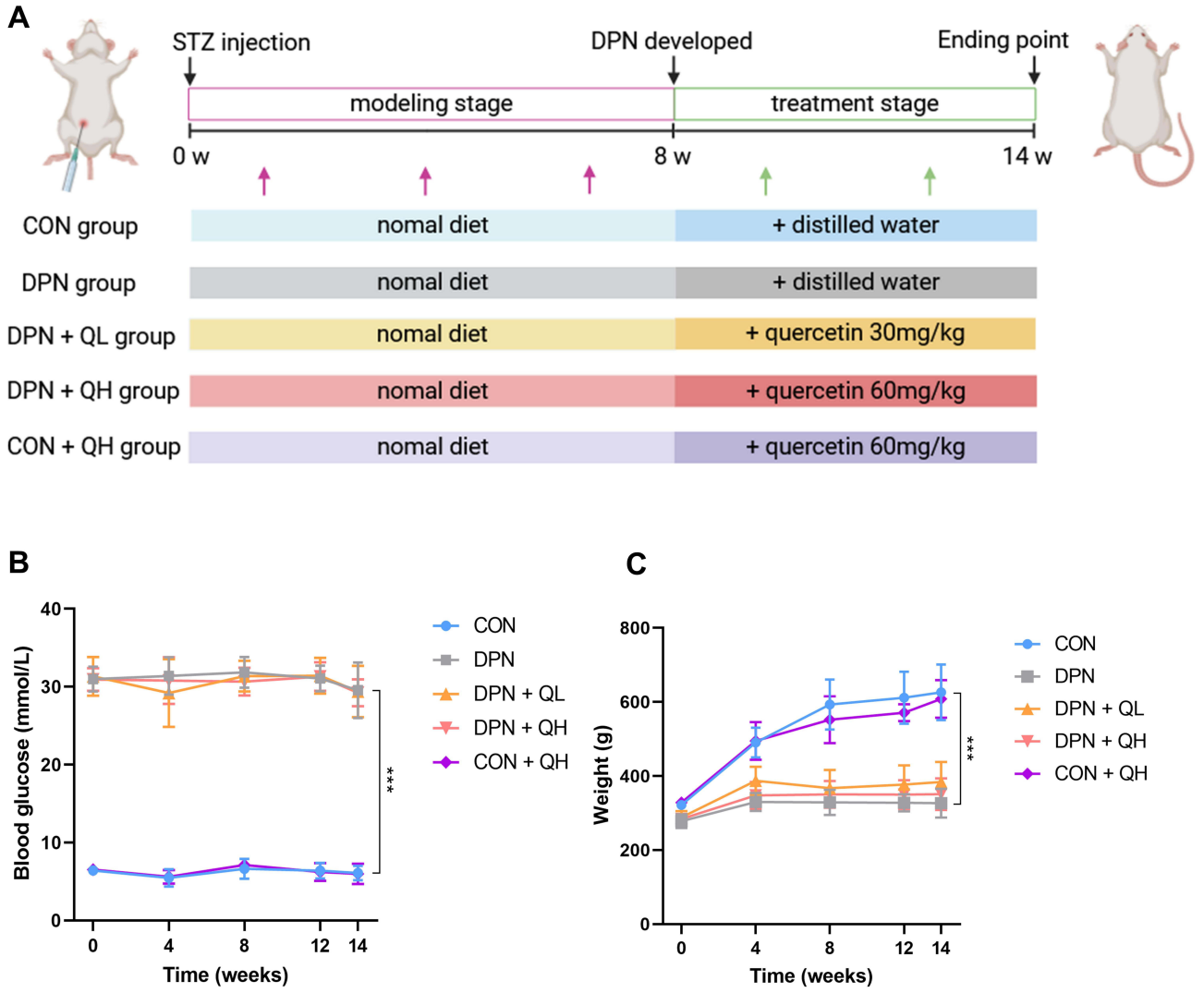



**Figure 1** Effects of quercetin on blood glucose and body weight of DPN rats. (**A**) The flowchart of the animal experiment. The blood glucose level (**B**) and body weight (**C**) of each group were monitored at 0 w, 4 w, 8 w, 12 w and 14 w, respectively. Data are expressed as mean ± SD. ***$p < 0.001$.
**Abbreviations**: CON, control; DPN, diabetic peripheral neuropathy; QL, quercetin at 30 mg/kg; QH, quercetin at 60 mg/kg.

## Blood Glucose and Body Weight

Quercetin administered at two different doses did not have a significant impact on blood glucose (Figure 1B, Supplementary Figure S4) or body weight (Figure 1C) of STZ-induced rats. Additionally, there were no significant differences in blood glucose and body weight between the CON group and CON + QH group.

## Effects of Quercetin on Serum Biochemical and Inflammatory Markers

To evaluate the safety of quercetin on DPN and healthy control rats, the serum levels of ALT, ALB, AST, and CREA were measured across different groups. The results indicated that quercetin administered at two different doses did not affect liver function since it has no impact on the levels of ALT and AST in serum (Figure 2A and B). Interestingly, quercetin markedly elevated the ALB levels of DPN rats (Figure 2C), while simultaneously reducing their CREA level (Figure 2D). Compared to the CON group, serum levels of IL-6 and IL-1β were notably elevated in DPN rats but were reduced following quercetin treatment (Figure 2E and F). Meanwhile, the eNOS levels were significantly decreased in the DPN group but were subsequently increased following quercetin treatment (Figure 2G). Additionally, there was no significant difference in the levels of these serum markers between the CON group and the CON + QH group.

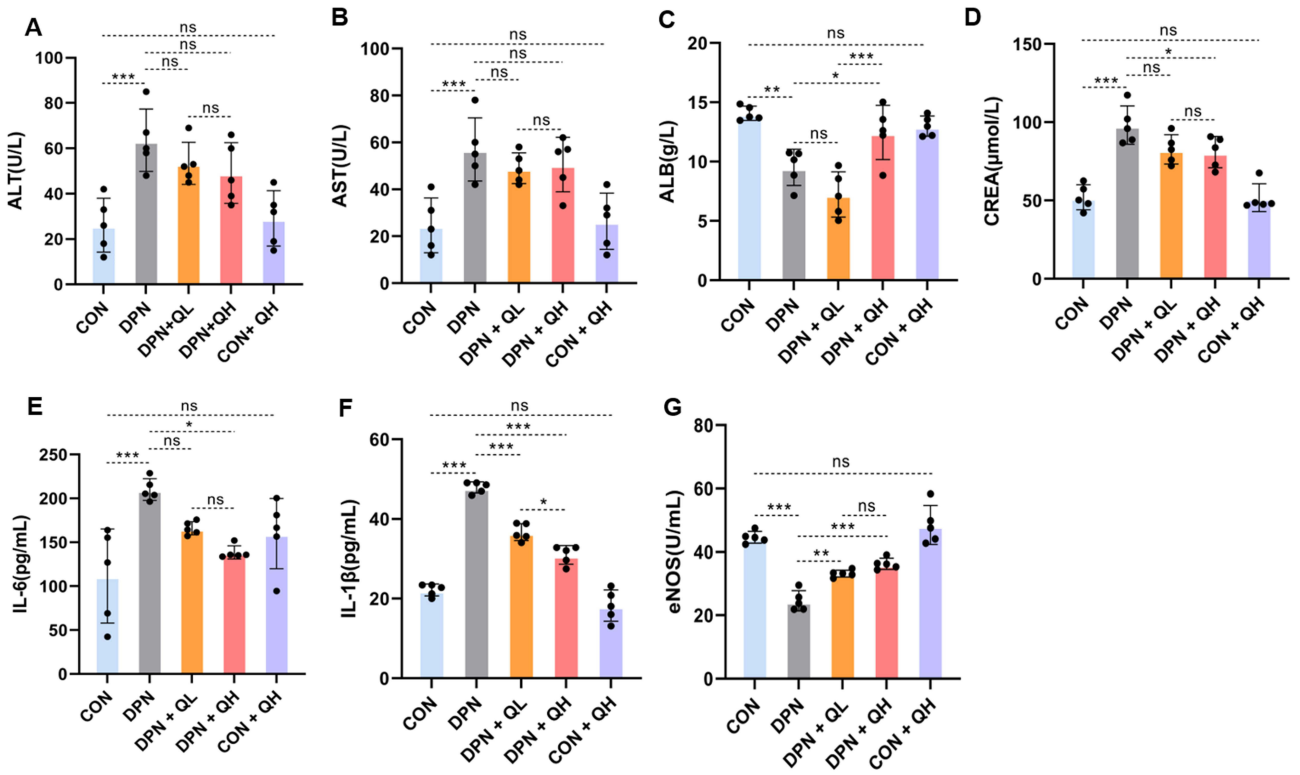



**Figure 2** Effects of quercetin on serum biochemical and inflammatory markers in DPN rats. (**A**) Alanine aminotransferase (ALT). (**B**) Aspartate aminotransferase (AST). (**C**) Albumin (ALB). (**D**) Creatinine (CREA). (**E**) Interleukin-6 (IL-6). (**F**) Interleukin-1β (IL-1β). (**G**) Endothelial Nitric Oxide synthase (eNOS). Data are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

**Abbreviations**: ns, no significant difference. CON, control; DPN, diabetic peripheral neuropathy; QL, quercetin at 30 mg/kg; QH, quercetin at 60 mg/kg.

## Effects of Quercetin on Peripheral Neurological Functions of DPN Rats

The impact of quercetin on peripheral neurological functions in DPN rats was evaluated using behavioral tests and MNCV measurement. The mechanical pain threshold was significantly lower in DPN group compared to the CON group. However, the mechanical pain thresholds in the DPN + QL and DPN + QH groups were significantly increased compared to the DPN group, with no significant difference observed between the two quercetin-treated groups (Figure 3A). The tail flick latency was significantly prolonged in the DPN model compared to healthy controls. Quercetin treatment at two different doses significantly shortened the tail flick latency compared to the DPN group, with the high dose showing a greater improvement than the low dose (Figure 3B). Additionally, the MNCV of DPN rats treated with both doses of quercetin significantly increased, although no significant difference was found between the two doses (Figure 3C). These results indicated that quercetin improved the peripheral neurological functions of DPN rats.

## Quercetin Alleviated Ultra-Pathologic Changes in Sciatic Nerves of DPN Rats

To investigate the impact of quercetin on neuropathologic ultrastructural changes in rats with DPN, sciatic nerve cross-sections were examined using TEM. The axons within the CON group displayed a consistent and complete morphology, characterized by a dense arrangement and uniform distribution of the myelin sheath, resulting in a clear layered structure (Figure 3D and I). Conversely, the axons of the sciatic nerves in the DPN group demonstrated varying levels of atrophy, irregular and disrupted myelin sheath structure, distinct layers, and numerous vacuoles. Demyelination and axon atrophy were prominently observed in DPN rats (Figure 3E and J). In comparison to the DPN group, both the DPN + QL and DPN + QH groups showed reductions in axonal degeneration, myelin vacuolization, and lamellar separation (Figure 3F, G, K, and L). Furthermore, the DPN + QH group demonstrated a more pronounced alleviation in demyelination. There was no significant distinction between the CON + QH (Figure 3H and M) and CON groups.

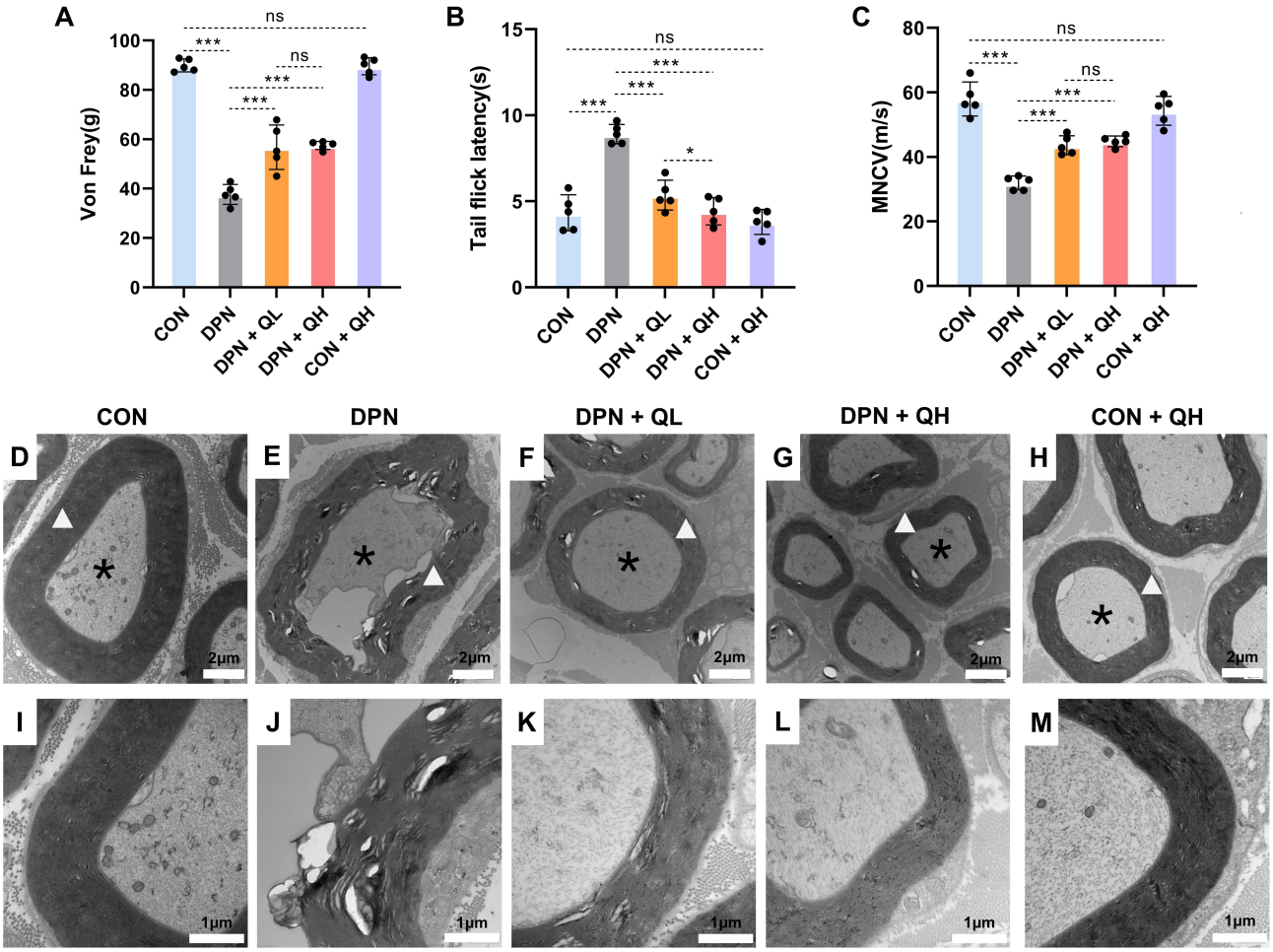



**Figure 3** Effects of quercetin on peripheral nerve function in DPN rats. The mechanical pain threshold (Von Frey) (**A**), tail flick latency (**B**) and MNCV (**C**) were detected at the 14 w of the experiment. Figure D-M shows the sciatic nerve of each group of rats observed using transmission electron microscopy (TEM). Above panel (**D**–**H**) shows 10,000× magnification (Scale bars = 2 μm) and below panel (**I**–**M**) shows 30,000× magnification (Scale bars = 1 μm). Triangle (Δ) indicates the myelin sheath, and the star(*) indicates the axon. Data are expressed as mean ± SD. *$p < 0.05$; ***$p < 0.001$.

**Abbreviations**: ns, no significant difference. CON, control; DPN, diabetic peripheral neuropathy; QL, quercetin at 30 mg/kg; QH, quercetin at 60 mg/kg.

## The Effect of Quercetin on Slit-2 in the Sciatic Nerve of DPN Rats

The protein expression of Slit-2 in the sciatic nerve was further analyzed using immunofluorescence. As depicted in Figure 4, a significant decrease in Slit-2 was observed in the DPN group compared to the CON group. Conversely, the DPN + QL and DPN + QH groups showed a marked increase in Slit-2 levels compared to the DPN group. No significant difference was found between the DPN + QL and DPN + QH groups, as well as between the CON and CON + QH groups.

## Quercetin Inhibited the Rho/ROCK Pathway in the Sciatic Nerve of DPN Rats

Figure 5 illustrates the immunohistochemical images of the sciatic nerve in various experimental groups. In comparison to the CON group, the expression levels of RhoA, ROCK2, and LIMK1 were significantly higher in the DPN group. Administration of quercetin at two doses resulted in decreased expression of RhoA, ROCK2, and LIMK1. Particularly, the expression of ROCK2 in the DPN + QH group was significantly lower than that in the DPN + QL group. No significant differences were observed in the expression levels of RhoA, ROCK2, and LIMK1 between the CON group and the CON + QH group. These above results demonstrated that quercetin inhibited the abnormal activation of the Rho/ROCK signaling pathway in sciatic nerves of DPN rats.

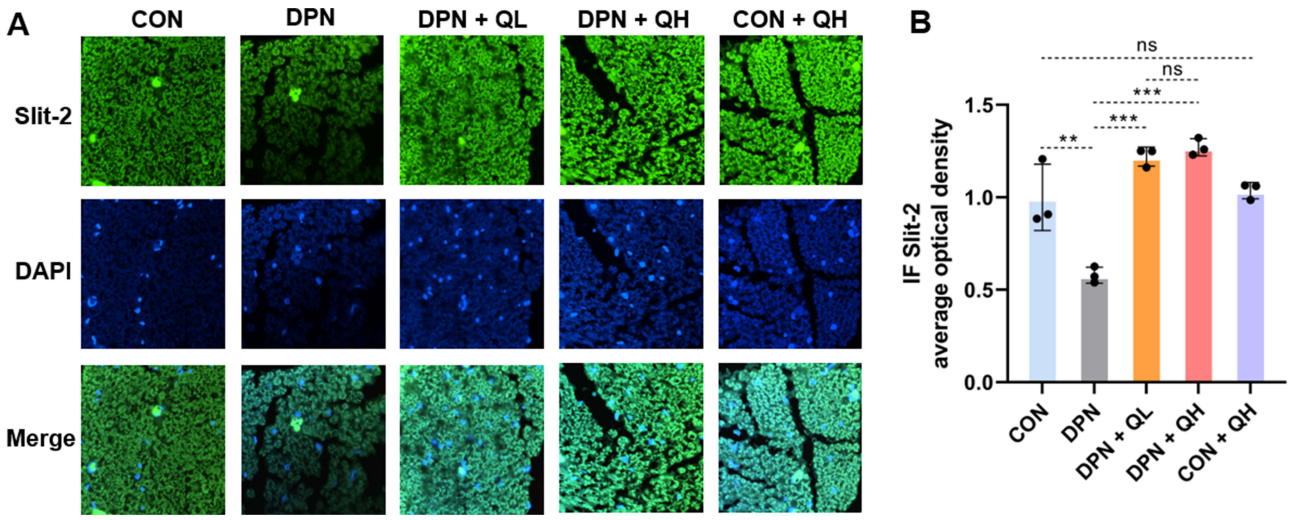



**Figure 4** The effects of quercetin on the expression of Slit-2 in sciatic nerve were detected by immunofluorescence assay. (**A**) The representative images of Slit-2. (**B**) The relative expression of Slit-2. Data are expressed as mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$.
**Abbreviations**: ns, no significant difference. CON, control; DPN, diabetic peripheral neuropathy; QL, quercetin at 30 mg/kg; QH, quercetin at 60 mg/kg.

## In-Vitro Studies

The in-vitro experiments were conducted to further investigate the mechanisms behind the neuroprotective effect of quercetin using rat Schwann cells RSC96 after high glucose stimulation. The protein levels of two axonal guiding factors and their receptors were evaluated.

### Effects of Quercetin on Netrin-1 and Its Receptors in HG-Exposed RSC96 Cells

As shown in Figure 6, the Netrin-1 level was significantly decreased under high glucose condition compared to the control group, whereas its receptors DCC and UNC5A showed opposite trends. Following quercetin intervention, the expression of Netrin-1 showed a marked increase, while the expression of UNC5A was significantly reduced in HG-exposed RSC96 cells. Similarly, fasudil, a ROCK inhibitor, also increased Netrin-1 expression and reduced UNC5A expression of HG-exposed RSC96 cells. Notably, DCC, another receptor of Netrin-1, remained unaffected by either quercetin or fasudil.

### Effects of Quercetin on Slit-2 and Its Receptor in HG-Exposed RSC96 Cells

As depicted in Figure 7, Slit-2 protein levels were significantly reduced in the HG group, whereas the Robo1 levels were markedly increased. The reduction in Slit-2 and elevation in Robo1 under HG conditions, was remarkably reversed by quercetin, as well as fasudil.

These data in vivo and in vitro collectively suggested that quercetin may function as a ROCK inhibitor, enhancing the expression of axon guidance factors, and thus protect Schwann cells from HG injury.

# Discussion

This study demonstrated that quercetin exhibits neuroprotective effects on streptozotocin-induced DPN rats. Treatment with quercetin for six weeks led to marked improvements in nerve function and alleviated pathological changes in the sciatic nerve. Furthermore, our findings suggest that quercetin modulates axonal growth factors both in vivo and in vitro. Additionally, quercetin inhibited the abnormal activation of the Rho/ROCK signaling pathway associated with DPN, suggesting a potential mechanism for quercetin's regulation of axonal guidance.

It is well known that the pathogenesis of DPN is complex.[26,27] Following peripheral nerve injury, the survival and apoptosis of neurons, as well as the reconstruction of axonal structures, constitute the structural basis for nerve repair and regeneration. Local microenvironments, such as Schwann cells, neurotrophic factors, and extracellular matrix, play a crucial role in nerve regeneration. Within this microenvironment, a complex interplay of guiding factors selectively

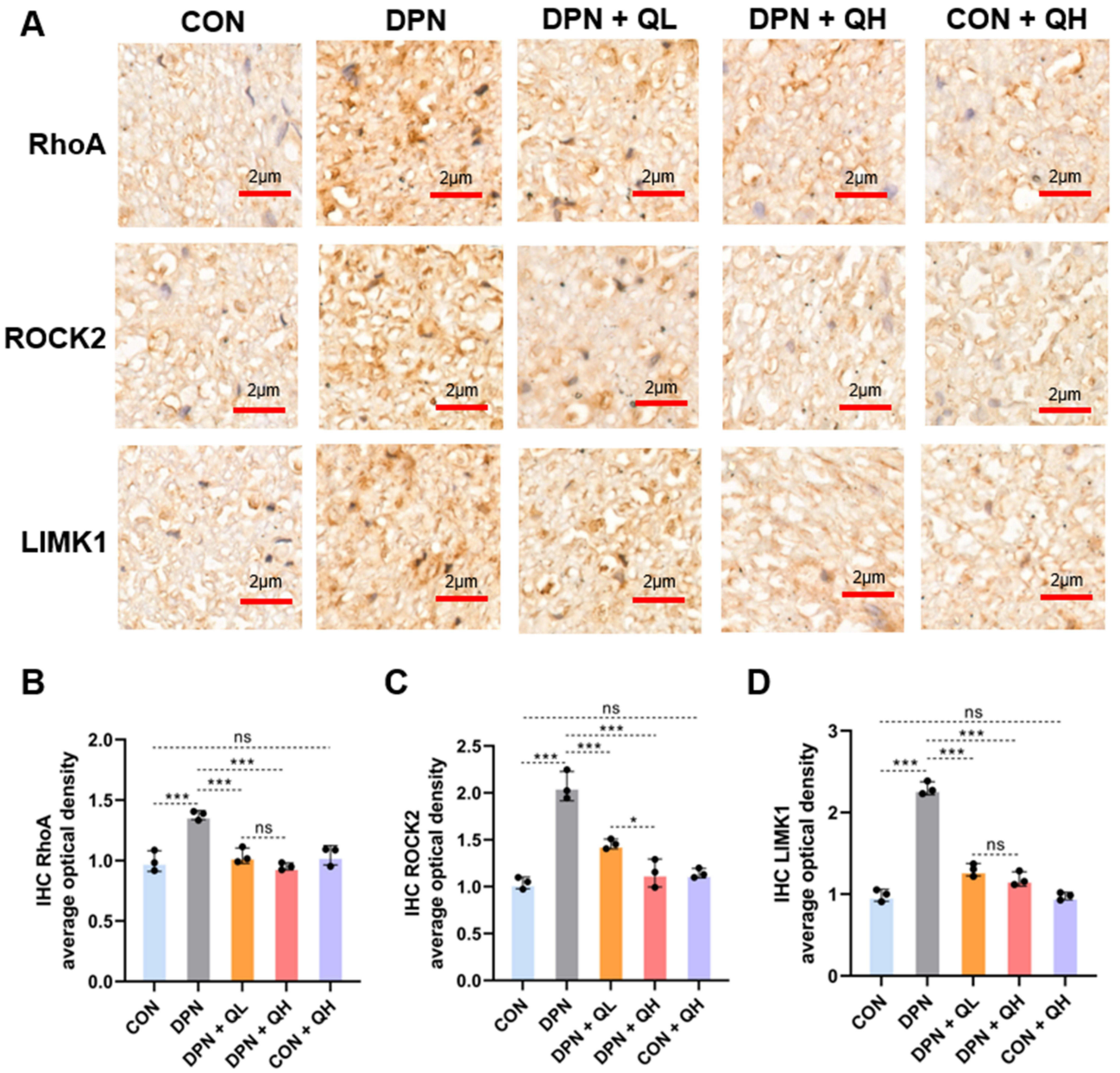



**Figure 5** Quercetin inhibited the activation of Rho/ROCK pathway in the sciatic nerve of DPN rats. (**A**) The immunohistochemical images. (**B**–**D**) The relative expression of RhoA, ROCK2, and LIMK1, respectively. Data are expressed as mean ± SD.*$p < 0.05$; ***$p < 0.001$.
**Abbreviations**: ns, no significant difference. CON, control; DPN, diabetic peripheral neuropathy; QL, quercetin at 30 mg/kg; QH, quercetin at 60 mg/kg.

attracts and repels axon growth cones, directing them towards target tissues and enabling precise reinnervation of injured areas. This process, crucial for nerve regeneration, is regulated by axonal guidance factors. Previous research has identified four families of proteins associated with axonal guidance molecules, specifically Netrins, Slits, Semaphorins, and Ephrins.[28] They influence axonal attraction or repulsion through binding to specific receptors located on the surface of axonal growth cones, thereby ensuring precise guidance of axon growth.[5]

Schwann cells are the main glial cells in peripheral nerve tissue. After peripheral nerve injury, Schwann cells transform into reparative phenotypes, proliferate, differentiate, envelop axonal structures, and secrete neurotrophic factors to promote the repair of damaged nerves. After peripheral nerve injury, Schwann cells migrate along newly regenerated blood vessels in the nerve gap from the proximal and distal nerve stumps, thereby guiding axonal regeneration.[29,30] If there is no guidance from Schwann cells in the nerve gap, the regenerated axons not only experience

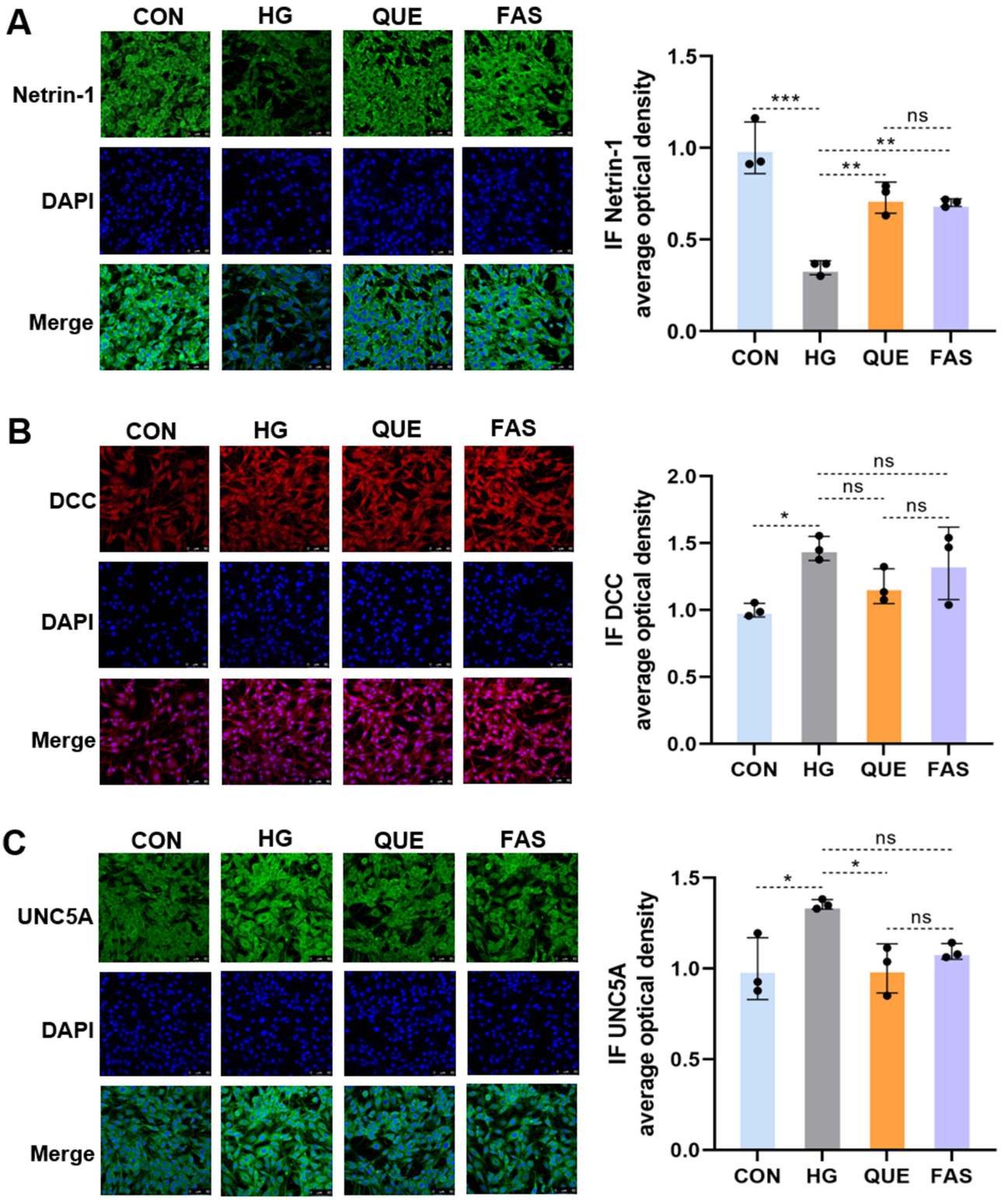



**Figure 6** Immunofluorescence assay of Netrin-1, DCC and UNC5A in RSC96 cells. The representative images and semi-quantitative analysis of Netrin-1 (**A**), DCC (**B**), and UNC5A (**C**) protein expression (scale bar = 50 μm, n=3). Data are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
**Abbreviations**: ns, no significant difference. CON, normal glucose; HG, high glucose; QUE, quercetin; FAS, fasudil.

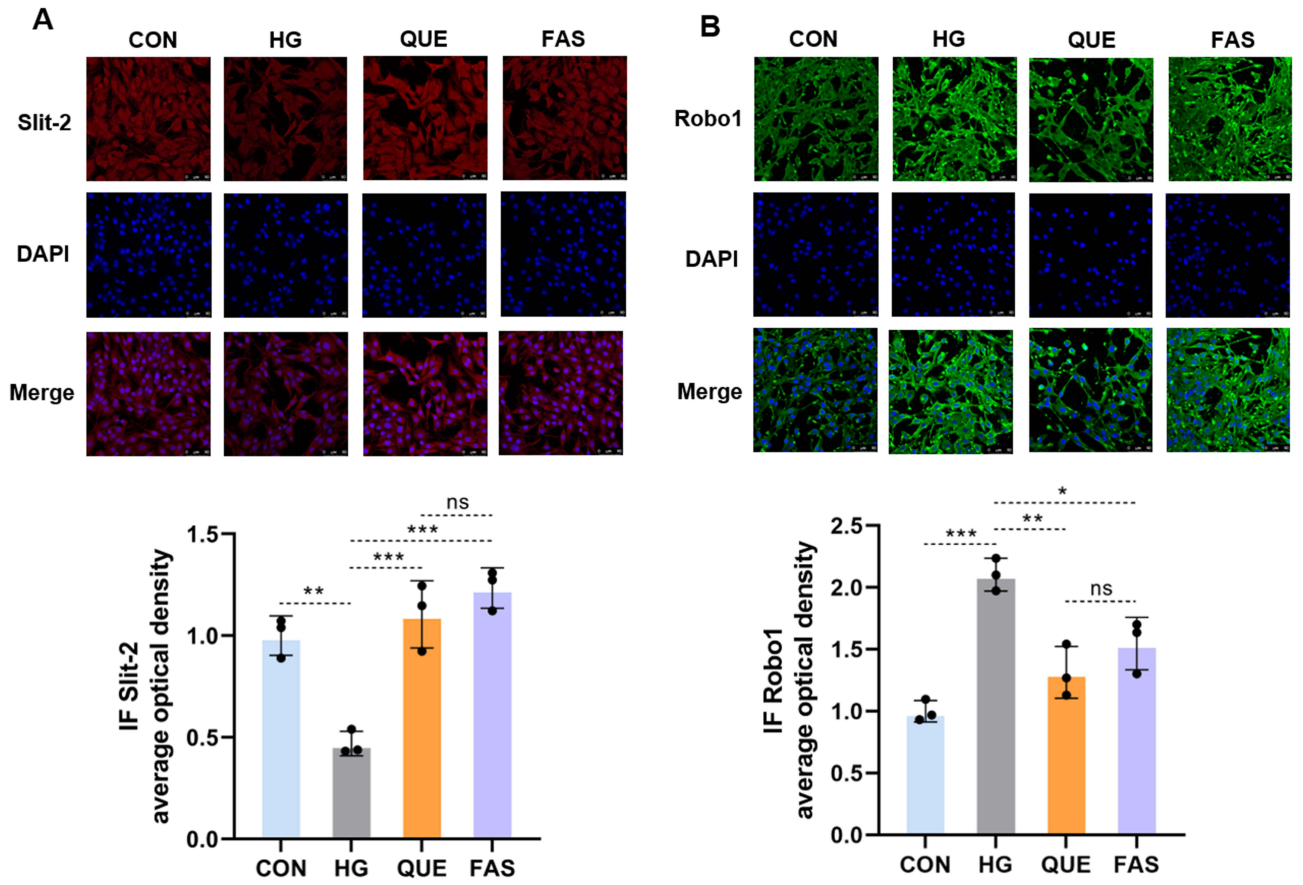



**Figure 7** Immunofluorescence assay of Slit-2 and Robo1 in RSC96 cells. The representative images and semi-quantitative analysis of Slit-2 (**A**) and Robo1 (**B**) protein expression (scale bar = 50 μm, n=3). Data are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
**Abbreviations**: ns, no significant difference. CON, normal glucose; HG, high glucose; QUE, quercetin; FAS, fasudil.

significant reduction in growth rate, but also lose directionality and fail to traverse the injured area, resulting in the inability to recover nerve function.[31]

Schwann cells can produce axon guidance factors to guide the regeneration of axons through nerve gaps. Classic axon guidance molecules such as Netrin-1 and Slit-2 are mainly expressed by Schwann cells. Among them, Netrin-1 is the prototypical axon guidance factor and a milestone in the field of axonal guidance research.[32,33] It can mediate axonal attraction or repulsion by binding to its receptors DCC and UNC5A.[34] Slit-2 is also a major axon guidance molecule that mediates repulsive signaling after binding to Robo receptors. Research has confirmed that Netrin-1 is upregulated after peripheral nerve injury.[29,31,35,36] Transplanting bone marrow mesenchymal stem cells overexpressing Netrin-1 to the site of sciatic nerve injury in rats can promote axonal regeneration and functional recovery.[37] After spinal cord injury, Slit-2 decreases in surviving neurons in the spinal cord, while Robo1 and RhoA increase, inhibiting the activity of growth cones and hindering the formation of new synapses in surviving neurons near the site of spinal cord injury. By enhancing Slit-2 activity or inhibiting RhoA, severe motor dysfunction induced by spinal cord injury can be reversed.[38] Our study showed that the expression of Slit-2 was significantly reduced in the sciatic nerve of DPN rats, and the level in Schwann cells under high glucose conditions was also significantly reduced. After intervention with quercetin, the expression of Slit-2 was upregulated. This suggests that quercetin can promote the secretion of axon directing factors by Schwann cells, and thus play a role in neural repair.

The Rho/ROCK signaling pathway consists of Rho GTPase, ROCK, and downstream substrates. Rho is a Ras-related GTP enzyme belonging to the Ras superfamily, which acts as a "molecular switch" by switching between GDP binding state (inactive state) and GTP binding state (active state).[39] ROCK is currently the most extensively studied primary downstream effector molecule of Rho, mainly including two subtypes: ROCK1 and ROCK2.[40,41] The Rho/

ROCK signaling pathway participates in various cellular behaviors and functions, including proliferation, differentiation, contraction, adhesion, migration, and apoptosis.[8] Research shows that RhoA/ROCK signaling pathway is critically involved in the pathogenesis of DPN and other chronic complications of diabetes.[10,42,43] High glucose levels can activate RhoA via multiple mechanisms mediated by protein kinase C (PKC) and reactive oxygen species, as well as directly activate ROCK.[44–47] Rho kinase enhances the phosphorylation of insulin substrate 1 (IRS-1), leading to the development of insulin resistance, while blocking the RhoA/ROCK signaling pathway can improve insulin resistance.[48,49] Pain hypersensitivity and allodynia is the main clinical manifestation of DPN, and some reports also indicate that the RhoA/ROCK pathway is involved in pain hypersensitivity and is central to the development and persistence of chronic pain.[50,51] Intramyelin injection of lysophosphatidic acid activates the RhoA/ROCK pathway to produce long-term thermal hyperalgesia, while ROCK inhibitor H-1152 can block thermal hyperalgesia in animals with partial sciatic nerve ligation.[50,51] After DPN causes peripheral nerve damage, RhoA activation subsequently activates ROCK, inducing phosphorylation of various target proteins, including myosin light chains (MLC) and LIMK, resulting in enhanced myosin contraction and collapse of growth cones.[52–54] The growth cone can guide the extension of the cytoskeleton to form axons, playing a crucial role in neuronal growth.[55] In conclusion, in the environment of diabetes, the Rho/ROCK pathway is activated, which mediates the assembly of MLC and actin, leading to the rearrangement of actin and microtubules in the growth cone, and ultimately causing growth cone collapse and arresting axonal elongation.[56] Our study found that the mechanical pain threshold of rats in the DPN group decreased, tail flick latency extended, and MNCV decreased, exhibiting hallmark features of DPN. In addition, the expression of RhoA, ROCK2, and LIMK1 in the sciatic nerve of rats in the DPN group significantly increased. Although quercetin's protective effect on DPN and its effect on the Rho/ROCK pathway have been proved, the direct target and mode of action ill defined. The next step should focus on finding the direct target and mechanism of quercetin in regulating Rho/ROCK pathway and protecting against DPN. In addition, the downstream proteins or enzymes involved in this process should be explored further.

Drawing from our team's prior experience, we determined a 6-weeks treatment of quercetin.[23,25] This study comprised five experimental groups (CON, DPN, DPN + QL, DPN + QH, CON + QH), one objective of which was to assess the potential side effects or toxicity of quercetin at higher dose. The findings indicate that high-dose quercetin (60 mg/kg) showed no effect on the liver function and kidney function of rats ($P>0.05$). Nevertheless, we have realized that the potential risks of long-term use of quercetin, especially to diabetic patients, merit further in-depth evaluation. Although this study shed light on the neuroprotective effect and mechanism of quercetin, one shortcoming of this study lies in the limited number of animals. Furthermore, due to the variations in the bioavailability of quercetin in different models or different species during oral administration, further study on its pharmacokinetics as well as the optimal dosage on DPN model is still necessary. In addition, a more comprehensive dose-response analysis could provide clearer insights into the optimal therapeutic dosage. The current doses do not seem to have reached the plateau of pharmacological effects. Therefore, in future studies, we will further expand the dose range based on this study and conduct further dose screening and optimization.

The current study evaluated the peripheral neuroprotective effect of quercetin on DPN, while the therapeutic effects of other flavonoids on DPN were not compared. Naturally occurring flavonoids include different structural types, including flavonols, dihydroflavones, isoflavones, chalcones, etc. They have some similar biological activities, but also have some structure-specific biological activities. Quercetin is a representative component of flavonols. Our study shows that quercetin can regulate the Rho/ROCK signaling pathway, but whether this activity is related to the hydroxyl group in its structure has not been reported. Therefore, in future research, on the one hand, we need to find the direct target of quercetin and its mechanism of regulating Rho/Rock signaling; on the other hand, it is also necessary to evaluate and compare the effect and mechanism of different types of flavonoids on DPN. This approach will allow for a comparative analysis to help identify potentially more effective compounds.

## Conclusion

This study has demonstrated that quercetin significantly alleviated STZ-induced DPN in a rat model. This effect is likely mediated through its ability to modulate axon guidance factors and inhibit the Rho/ROCK signaling pathway. Overall, our findings suggest that quercetin may serve as a promising therapeutic candidate for DPN treatment.

## Abbreviations

DPN, Diabetic peripheral neuropathy; ROCK, Rho Kinase; MNCV, Motor nerve conduction velocity; Slit-2, Slit guidance ligand 2; STZ, streptozotocin; ALT, alanine aminotransferase; ALB, albumin; AST, aspartate aminotransferase; CREA, creatinine; IL-6, interleukin-6; IL-1β, interleukin-1β; eNOS, endothelial nitric oxide synthetase.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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