

Quercetin reduces inflammation in a rat model of diabetic peripheral neuropathy by regulating the TLR4/MyD88/NF- κ B signalling pathway

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ABSTRACT

Neuroinflammation contributes significantly to the pathogenesis of diabetic peripheral neuropathy (DPN). Quercetin reportedly exerts neuroprotective effects in DPN. Here, we aimed to evaluate the potential anti-inflammatory effects of quercetin in a DPN rat model. Eight weeks after streptozotocin administration, diabetic rats were treated with quercetin (30 and 60 mg/kg/day orally) for 6 weeks. We assessed the mechanical withdrawal threshold (MWT), nerve conduction velocity (NCV) and morphological changes in sciatic nerves. Additionally, we measured the levels of tumour necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6 by ELISA and the expression of TLR4, MyD88, and NF- κ B in sciatic nerves by western blotting and immunohistochemical assays. Our results revealed that blood glucose levels and body weight were unaltered following quercetin treatment. However, quercetin improved MWT ($p < 0.05$), NCV ($p < 0.05$), and pathological changes in the sciatic nerves of DPN rats. Quercetin significantly alleviated the increased expression of TNF- α ($p < 0.05$) and IL-1 β ($p < 0.001$). Furthermore, high-dose quercetin administration significantly downregulated the expression of TLR4 ($p < 0.001$), MyD88 ($p < 0.001$), and NF- κ B ($p < 0.001$) in sciatic nerves of DPN rats. Our findings revealed that quercetin could reduce the levels of inflammatory factors in DPN rats, possibly mediated via the downregulation of the TLR4/MyD88/NF- κ B signalling pathway. Collectively, these results suggest that although quercetin did not decrease blood glucose levels or reversed the reduced body weight, it showed anti-inflammatory and neuroprotective effects, which was beneficial for the treatment of DPN.

1. Introduction

In 2019, the global prevalence of diabetes mellitus (DM) was estimated to be 9.3% (463 million people) in the adult population and is predicted to exceed 10% in the next decade (Saeedi et al., 2019). Diabetic peripheral neuropathy (DPN), one of the most prevalent complications encountered in patients with diabetes, leads to impaired quality of life owing to neuropathic pain, sensory loss, limb amputation, and high mortality rates (Feldman et al., 2019; Liu et al., 2018).

Recent evidence suggests an association between long-term, low-grade neuroinflammation and DPN (Stino et al., 2020). Some clinical trials have reported that levels of inflammatory cytokines, including tumour necrosis factor (TNF)- α and interleukin (IL)-6, are higher in the sera of patients with DPN than in the control groups (Mu et al., 2017; Schlesinger et al., 2019). The inflammatory cascade triggered by inflammatory factors is known to damage the function and structure of peripheral nerves, including myelin damage and microangiopathy

(Sandireddy et al., 2014). Cytokines such as TNF- α , IL-1 and IL-6 promote sensitisation of the peripheral nervous system to produce neuropathic pain (Lees et al., 2017; Shi et al., 2013a).

Toll-like receptor 4 (TLR4) is a transmembrane pattern-recognition receptor that can be activated by multiple pathogen-derived and damage-related substances (Calvo-Rodriguez et al., 2020). TLR4 recruits myeloid differentiation factor 88 (MyD88) to activate nuclear factor kappa B (NF- κ B), promoting the transcription of inflammatory cytokines (Azam et al., 2019). The TLR4/MyD88/NF- κ B signalling pathway directly regulates cytokines that stimulate the inflammatory cascade reaction. Thus, it is considered a novel therapeutic target for neuroinflammation-related diseases (Rahimifard et al., 2017). Reports have suggested that inhibition of the TLR4 signalling pathway in the spinal cord could downregulate the levels of TNF- α , IL-1 β , and other inflammatory factors, resulting in improved nerve function, such as mechanical hyperalgesia in diabetic rat models with neuropathy (Liu et al., 2018).

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Quercetin, a natural flavonoid, is extensively distributed in various fruits and vegetables (Shi et al., 2019). Accumulating evidence has shown that quercetin has neuroprotective effects and prevents neurodegenerative diseases by inhibiting neuroinflammation (Spagnuolo et al., 2018). Quercetin reportedly exerts a neuroprotective function in hypoxia-ischaemia-induced brain injury by modulating the TLR4/NF- κ B signalling pathway (Wu et al., 2019). In addition, some reports have focused on the neuroprotective effects of quercetin on DPN. Quercetin treatment can attenuate thermal hyperalgesia, cold allodynia, and mechanical allodynia in DPN animal models (Yang et al., 2019; Anjaneyulu and Chopra, 2004). Therefore, whether quercetin plays a neuroprotective role in DPN by mediating anti-inflammatory effects needs to be further explored. In addition, the potential role of quercetin in targeting the TLR4/NF- κ B signalling pathway to alleviate DPN remains unclear.

In the current study, we aimed to verify the neuroprotective and anti-inflammatory effects of quercetin in a streptozotocin (STZ)-induced DPN rat model. Additionally, we examined the potential underlying mechanisms by assessing its effect on the TLR4/MyD88/NF- κ B signalling pathway to determine whether quercetin can be employed as an efficacious and feasible candidate for treating DPN.

2. Materials and methods

2.1. Animal model and drug administration

Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research. Ethical approval for this study was granted by the Institutional Animal Care and Use Committee of the Peking Union Medical College Hospital (application no. XHDW-2021-00). Male Sprague-Dawley rats weighing 200–220 g were provided by Vital River Laboratory Animal Technology (Beijing, China). All animals were housed in the Experimental Animal Centre of the Peking Union Medical College Hospital (Beijing, China), maintained in standard rodent cages under artificial light (12 h light: 12 h dark cycles) at room temperature ($21 \pm 1^\circ\text{C}$) and humidity (40–70%), and fed a chow diet *ad libitum* and filtered tap water.

After an overnight fast, the diabetic rat model was established by administering a single intravenous dose of 60 mg streptozotocin (Sigma Aldrich, USA)/kg body weight in 0.1 mol/L citrate buffer (pH4.5); the non-diabetic control animals were administered with the same volume of citrate buffer. Fasting blood glucose levels were measured using blood from the tail tip with a blood glucometer (Roche, Ireland) 72 h after injection. The diabetic model was successfully established if blood glucose concentrations exceeded 16.7 mmol/L (Furman, 2015).

It has been established that the DPN phenotype in rats develops 8 weeks after diabetes induction (Biessels et al., 2014). Therefore, 8 weeks after STZ injection, the diabetic model rats were randomly divided into three groups ($n = 5$ per group): the DPN model group, the low-dosage quercetin-treated group administered 30 mg/kg/day (DPN + Q30), and the high-dose quercetin-treated group administered 60 mg/kg/day (DPN + Q60). Subsequently, the DPN + Q30 and DPN + Q60 groups were intragastrically administered 30 and 60 mg/kg/day quercetin for 6 weeks. Quercetin used for gavage was freshly prepared daily. First, quercetin powder (Sigma Aldrich, USA) was added to distilled water and mixed evenly by shaking. The rats were then immediately administered quercetin intragastrically. Meanwhile, 5 age-matched healthy rats with blood glucose <7.0 mmol/L in the control group (CON) and rats in the DPN model group received the same volume of distilled water. In addition, body weight and blood glucose levels were monitored during the observational period.

After 6 weeks of treatment, all rats were euthanised by intraperitoneal injection of 10% chloral hydrate (3 mL/kg) and sacrificed. Blood samples collected from the carotid artery were centrifuged at 3000 rpm for 15 min, and serum specimens were stored at -80°C . Subsequently,

sciatic nerves were harvested from the left side and cut into two sections: one fixed in 10% phosphate-buffered formalin for immunohistochemical and histological examination; the other was fixed in 2.5% glutaraldehyde for electron microscopy. The right sciatic nerves were immediately frozen in liquid nitrogen and stored at -80°C for western blot analysis. In addition, dorsal root ganglia (DRG) were harvested and fixed in 10% phosphate-buffered formalin for haematoxylin and eosin (HE) staining.

2.2. Mechanical withdrawal threshold

To examine mechanical hyperalgesia, the mechanical pain threshold was determined using the Von Frey Pain Measurement Instrument (ITC Life Science Inc., USA) after 6 weeks of drug administration. Briefly, transparent glass cells were placed on a mesh metal plate 50 cm from the experimental bench. The rats were habituated in individual cells for 15 min. Then, the electronic von Frey filament vertically stimulated the plantar surface of the hind paw. The value at which the hind paw was abruptly withdrawn was recorded as a positive response. Each rat was evaluated three times with the von Frey filament to assess mechanical hyperalgesia (Zhang et al., 2020).

2.3. NCV in STZ-induced diabetic rats

After treatment with quercetin for 6 weeks, motor nerve conduction velocity (NCV) was measured in rat sciatic nerves using an EMG/EP system (NCC Medical Co. Shanghai, China). Under anaesthesia, the rat legs were shaved prior to measurement. The body temperature of rats was maintained between 37.5 and 38.5°C using a heating lamp. The bipolar active electrode P1 was placed in the sciatic notch of the sciatic nerve and functioned as the proximal portion. The bipolar recording electrode P2 was pierced the tibial notch of the tibial nerve as the distal point, and the ground electrode was situated at the root of the rat tail. After an electrical stimulus, the amplitude and latency (t) were obtained using an EMG machine. Simultaneously, the length (l mm) between P1 and P2 was determined using a calliper. NCV was calculated by dividing the length (l) by the latency (t) (Xie et al., 2020).

2.4. HE and toluidine blue staining

Briefly, sciatic nerves and DRG were harvested and placed in 10% phosphate-buffered formalin for fixation. After dehydration with ethanol at a gradient degree, the samples were embedded in paraffin and sliced into $4\text{-}\mu\text{m}$ thick sections. Then, following deparaffinisation, sections were stained with HE dye solution. The histomorphology of sciatic nerves and DRG was examined using a Leica DM3000 microscope, and images were captured using a Leica image acquisition system (Leica Microsystems, Germany). Another section of the sciatic nerve was obtained and fixed in 10% phosphate-buffered formalin at 4°C . The sections were immersed in 1% osmium acid, dehydrated, embedded, and cut into $1\text{-}\mu\text{m}$ thick sections. Then, samples were stained with toluidine blue and observed under an optical microscope. The morphometric analysis was performed using Image-J 1.46 (National Institutes of Health, USA). The total number of myelinated axons and abnormal myelinated axons per $180 \times 135 \mu\text{m}^2$ were enumerated. Abnormal myelinated axons were characterised by axon atrophy, vacuolar changes, and myelin sheath fragmentation. Three rats from each group were included in the analysis. The percentage of abnormal myelinated axons (abnormal axons with disintegrated myelin/total axons) was calculated.

2.5. Electron microscopy

The sciatic nerve was processed for electron microscopy following routine transmission electron microscopy (TEM) imaging. In brief, the sciatic nerve was fixed in 2.5% glutaraldehyde for 2–4 h, rinsed in 0.1 M phosphate-buffered saline (PBS; pH 7.4), and then fixed in 1% osmium

tetroxide for 1 h. After dehydration using graded alcohol, the sample was embedded in epoxy resin and cured at 37, 45, and 60 °C. Next, 70-nm thick sections were cut with an ultramicrotome (Leica EM UC7, Germany) and mounted on 200 mesh copper TEM grids, which were then stained with uranyl acetate and lead citrate. Myelin sheaths and axons were observed using a transmission electron microscope (TEM-1400 plus, JEOL, Japan) at 10,000 × and 30,000 × magnification and captured using a CCD camera (OSIS, VELETA, Germany).

2.6. Enzyme-linked immunosorbent assay (ELISA)

To analyse biochemical parameters of inflammation, TNF- α , IL-1 β , and IL-6 in serum and sciatic nerves were determined using an enzymatic method with a commercial kit, according to the manufacturer's instructions. Briefly, samples and standards were transferred equally to the wells, which contained the same detection reagent, substrate solution, and stop solution, following the ELISA kit (Elabsience, China) instructions for washing and incubation. Then, the plate was measured with a microplate reader (DR-200Bs, Hiwell Diatek, China) at 450 nm optical density. Each sciatic nerve sample was washed with cold PBS and homogenised on ice. After centrifugation at 4 °C, the supernatants were transferred to 96-well plates. Total protein was measured using the Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Scientific, USA) using a microplate reader (Hiwell Diatek, China) at 562 nm. Protein concentration was quantified using a standard curve. The concentrations of TNF- α , IL-1 β , and IL-6 in the sciatic nerves were calculated using the following formula: concentration = measurement of ELISA (pg)/total protein (mg).

2.7. Immunohistochemical assay (IHC)

Briefly, isolated sciatic nerves were placed in 10% phosphate-buffered formalin, followed by dehydration with gradient alcohol. The samples were embedded in paraffin, sliced into 4- μ m thick sections, and placed on polylysine-coated slides. Slides were passed through xylol and graded ethanol series. Next, samples were placed in citrate buffer (pH 6.0) for 15 min to repair hot antigens, and the endogenous peroxidases were blocked with 3% H₂O₂ solution. The sections were incubated with primary antibodies against TLR4 (Abcam Plc, UK, ab217274, rabbit, 1:100), MyD88 (Abcam Plc, UK, ab219413, rabbit, 1:100), and p65 NF- κ B (Abcam Plc, UK, ab16502, rabbit, 1:100) at 4 °C overnight. After washing with PBS three times for 5 min, sections were incubated with a secondary antibody (ZSGB-BIO, China, DS-0001) at room temperature for 1 h. Then, the sections were developed with the diaminobenzidine (DAB) kit (Solarbio, China), following the manufacturer's instructions. The stained areas were observed and captured using a Leica DM3000 microscope and a Leica image acquisition system (Leica Microsystems, Germany). Image-J 1.46 was used to analyse the positive images.

2.8. Western blotting analysis

The sciatic nerves were harvested, cut, and homogenised in RIPA lysis buffer for protein extraction. The total protein concentration of each sample was determined using a Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Scientific, USA). Each sample containing the same amount of protein was subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore, USA). After blocking with 5% bovine serum albumin for 2 h, the blots were incubated with primary antibodies against TLR4 (Abcam Plc, UK, ab217274, rabbit, 1:300), MyD88 (Abcam Plc, UK, ab219413, rabbit, 1:500), p65 NF- κ B (Abcam Plc, UK, ab16502, rabbit, 1:1000), and β -tubulin (Abcam Plc, UK, ab6046, rabbit, 1:5000) at 4 °C overnight, followed by washing and incubation with the horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO, China, ZB-2301) for 1 h. Consequently, immunoblots were developed using enhanced chemiluminescence (ECL kit, Solarbio, China) and visualised

using a multi-functional imaging analysis system (GE AI680, USA). At levels comparable with the housing-keeping gene (β -tubulin), the relative optical density of each blot band was quantified and analyzed using Image-J 1.46.

2.9. Statistical analysis

Data analyses were performed using IBM SPSS Statistics 26 (USA) and GraphPad Prism 8 (USA). Data values are presented as the group mean $\pm$ standard deviation (SD). Statistical differences were determined using Student's t-test or one-way ANOVA, followed by Tukey's multiple-comparison test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Body weight and blood glucose levels

As shown in Fig. 1, after DM induction for 14 weeks, the STZ-induced diabetic rats showed increased blood glucose levels ($p < 0.001$) and decreased body weight ($p < 0.001$) when compared with normal control rats. However, no significant difference in blood glucose or body weight was observed between the quercetin-treated group and DPN model group.

3.2. Mechanical withdrawal threshold

The von Frey test was performed to determine the mechanical withdrawal threshold of experimental animals. As shown in Fig. 2A, the mechanical withdrawal threshold of the DPN group was lower than that of the CON group ($p < 0.001$). Simultaneously, quercetin treatment at 30 and 60 mg/kg (DPN + Q30 and DPN + Q60) improved the mechanical withdrawal threshold of DPN rats ($p < 0.05$). However, no statistically significant differences in the mechanical withdrawal threshold values were detected between the quercetin-treated groups ($p > 0.05$).

3.3. NCV in STZ-induced diabetic rats

As shown in Fig. 2B, after DM induction for 14 weeks, NCV of untreated diabetic rats significantly decreased when compared with the age-matched healthy rats ($p < 0.001$); this parameter was improved in quercetin-treated diabetic rats in both dosage groups (54.27 ± 4.57 m/s in CON group, 31.45 ± 3.26 m/s in DPN group vs. 43.12 ± 3.31 m/s in DPN + Q30 group, $p < 0.05$; 31.45 ± 3.26 m/s in DPN group vs. 45.55 ± 3.13 m/s in DPN + Q60 group, $p < 0.05$). However, the difference in NCV between the two quercetin-treated groups was not significant ($p > 0.05$). Moreover, no statistically significant difference was observed between the left and right sciatic nerves of rats within the same group.

3.4. Pathomorphological changes in sciatic nerves by light microscopy

HE- and toluidine blue-stained sections were prepared to observe pathomorphological changes in the sciatic nerves by light microscopy. As shown in Fig. 3, the cross (Fig. 3A) and longitudinal (Fig. 3B) HE-stained sections of sciatic nerves showed orderly arranged nerve fibres with an intact myelin sheath in the CON group. However, fibres were loose, disordered, less intensely stained, and differed in size in the DPN model group, exhibiting visible signs of axonal atrophy and myelin damage (green arrows). As shown in Fig. 3C, the HE-stained DRG of the CON group exhibited compact neurons with a clear structure. The granular Nissl bodies (blue arrows) were distributed in the cytoplasm of the neurons. Moreover, the neuronal structure was disorganised and loose in the DPN model group. Vacuolar changes, faded staining, and decreased Nissl bodies were observed in DRG neurons of the DPN group. These morphological changes in both sciatic nerves and DRG neurons were alleviated in quercetin-treated groups.

As shown in Fig. 4A and B, toluidine-stained sciatic nerve cross-

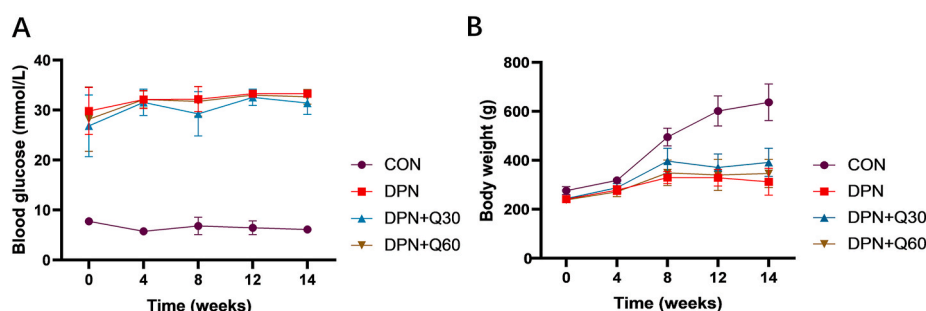


Fig. 1. Body weight and blood glucose levels after streptozotocin injection. After 8 weeks, streptozotocin-injected rats received quercetin (30 and 60 mg/kg/day orally) for 6 weeks ($n = 5$ per group). CON, control; DPN, diabetic peripheral neuropathy; Q30, quercetin at 30 mg/kg; Q60, quercetin at 60 mg/kg.

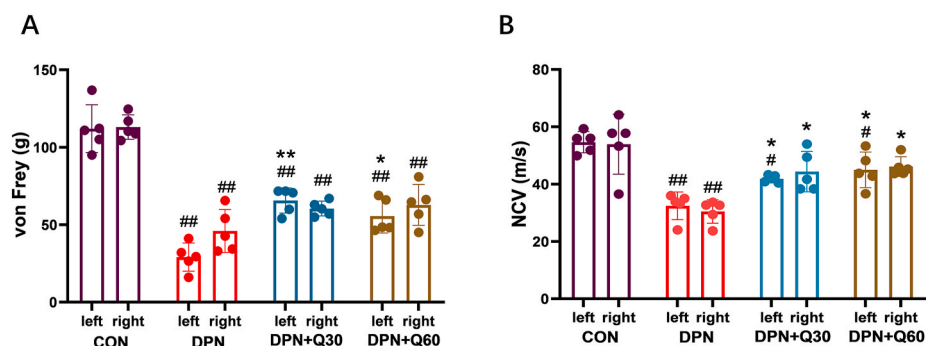


Fig. 2. Effect of quercetin on mechanical withdrawal threshold (von Frey) (A) and motor nerve conductivity velocity (NCV) (B). Data are expressed as mean $\pm$ standard deviation (SD) ($n = 5$ per group). ## $p < 0.001$, # $p < 0.05$ versus control rats and ** $p < 0.001$, * $p < 0.05$ versus DPN model rats by one-way ANOVA and Student's t -test. CON, control; DPN, diabetic peripheral neuropathy; Q30, quercetin at 30 mg/kg; Q60, quercetin at 60 mg/kg.

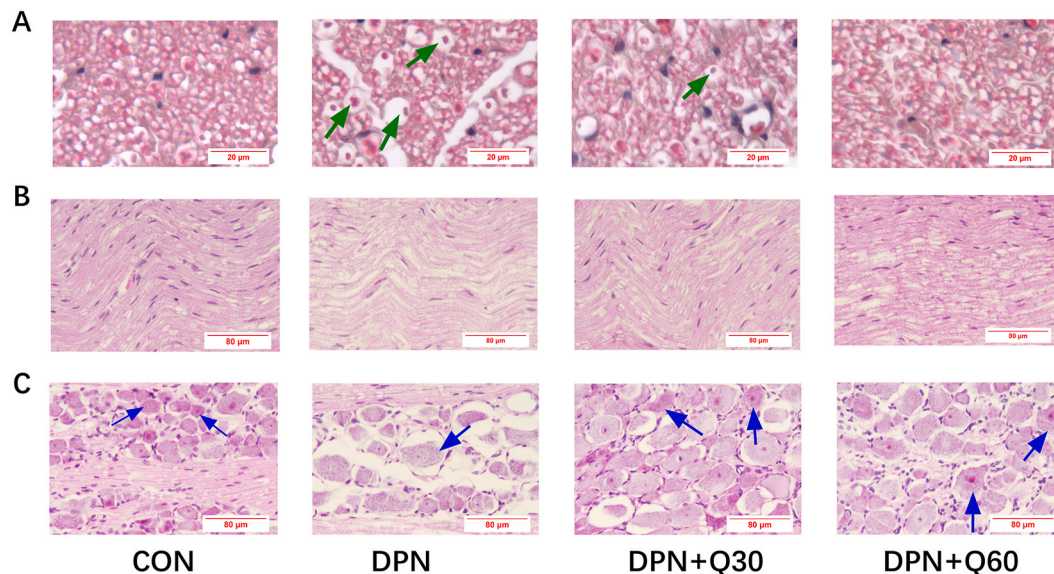


Fig. 3. Pathomorphological changes in sciatic nerves (SN) and dorsal root ganglia (DRG) by H&E staining. A: Cross-section of SN; B: longitudinal section of SN; C: longitudinal section of DRG. The SN of normal rats in the CON group exhibits orderly arranged nerve fibres with an intact myelin sheath. The SN of DPN model rats exhibits notably damaged myelin and axonal shrinkage (green arrows). The neuronal structure of DRG neurons is clear with granular Nissl bodies in the cytoplasm (blue arrows). Vacuolar changes, fading staining, and decreased Nissl bodies can be observed in DRG neurons of DPN model rats. These pathological changes in both SN and DRG are improved in the quercetin-treated groups (DPN + Q30 and DPN + Q60). The panel shows 400 $\times$ magnification (scale bar = 20 μ m, 80 μ m and 80 μ m, respectively). $n = 3$ per group. CON, control; DPN, diabetic peripheral neuropathy; Q30, quercetin at 30 mg/kg; Q60, quercetin at 60 mg/kg. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sections of the CON group exhibited a clear structure with thick axons surrounded by a uniform myelin sheath, proportional in thickness to their axons. In contrast, sparsely distributed myelinated fibres accompanied by obviously damaged myelin with axonal atrophy, vacuolar

changes, and myelin sheath fragmentation were observed in the DPN group. Compared with the DPN group, both the diameter and density of nerve fibres were increased in quercetin-treated groups. Moreover, axonal shrinkage and myelin damage were markedly reduced in the

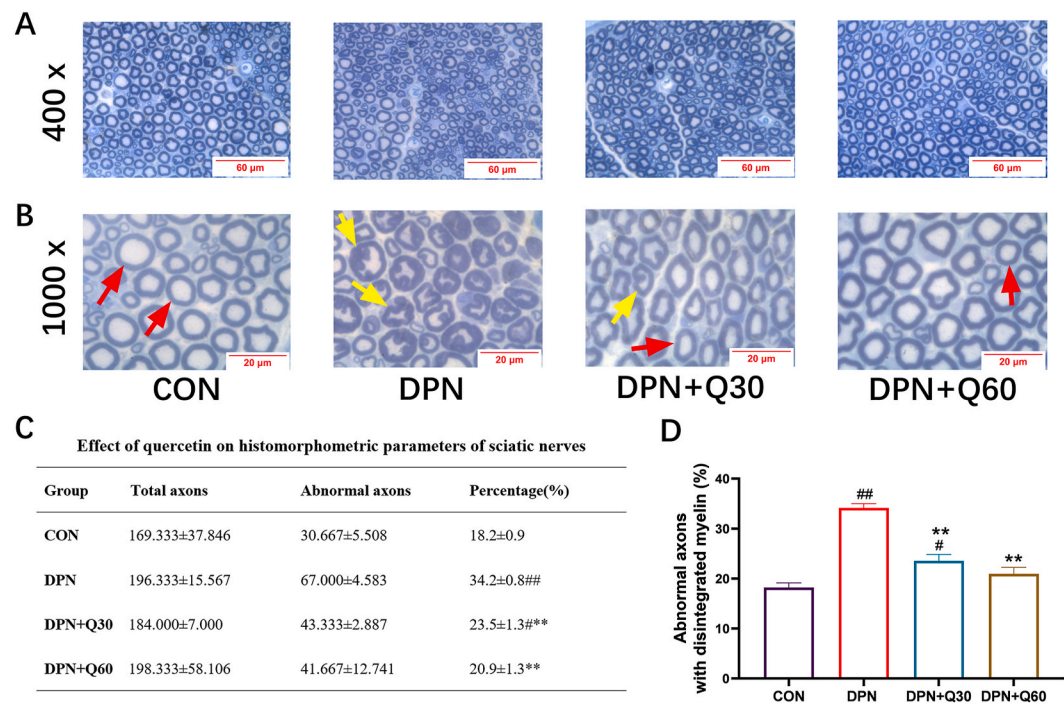


Fig. 4. Pathomorphological changes in sciatic nerves examined by toluidine blue staining. A and B: Representative images of toluidine blue-stained sciatic nerves. The left panel represents 400 × and 1000 × magnified images (scale bar = 60 μm and 20 μm, respectively). C and D: Total axons and abnormal axons with disintegrated myelin were enumerated in the same toluidine blue stained images. The percentage of abnormal axons with disintegrated myelin/total axons was calculated. The results are presented in a table and bar graph. The red arrows indicate normal myelinated nerve fibres (thick axons and uniform myelin). The yellow arrows indicate myelinated fibre degeneration (axonal atrophy and myelin sheath fragment). Data are expressed as the mean ± standard deviation (SD), n = 3. ##*p* < 0.001, #*p* < 0.05 versus control rats, and ***p* < 0.001 versus DPN rats by one-way ANOVA. CON, control; DPN, diabetic peripheral neuropathy; Q30, quercetin at 30 mg/kg; Q60, quercetin at 60 mg/kg. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

quercetin-treated groups. As shown in Fig. 4C and D, the semi-quantitative assessment of pathomorphological characteristics showed an increased percentage of abnormal axons with disintegrated myelin in the DPN group when compared with the CON group (*p* < 0.001). Treatment with quercetin at both doses (30 and 60 mg/kg) significantly decreased the percentage of abnormal axons with disintegrated myelin (*p* < 0.001); however, no significant difference was observed between the DPN + Q30 and DPN + Q60 groups.

3.5. Ultrastructural changes in sciatic nerves examined by TEM

Consistent with HE and toluidine blue staining results, ultrastructural changes in the sciatic nerves of DPN rats exhibited myelin

fragmentation and axon atrophy, as determined by TEM. As shown in Fig. 5, the regular round axons of myelinated nerve fibres were wrapped with a myelin sheath, exhibiting a clear lamellar structure. The most notable ultrastructural alterations in the DPN group were damaged myelin sheaths, characterised by bubble-like protrusions, axonal shrinkage, and separated lamellar space. Quercetin treatment at both doses improved these myelin sheath changes and ameliorated axonal atrophy. In particular, the high-dose group exhibited more obvious remission of myelin sheath damage.

3.6. Serum inflammatory markers

To further investigate whether inflammatory cytokines are

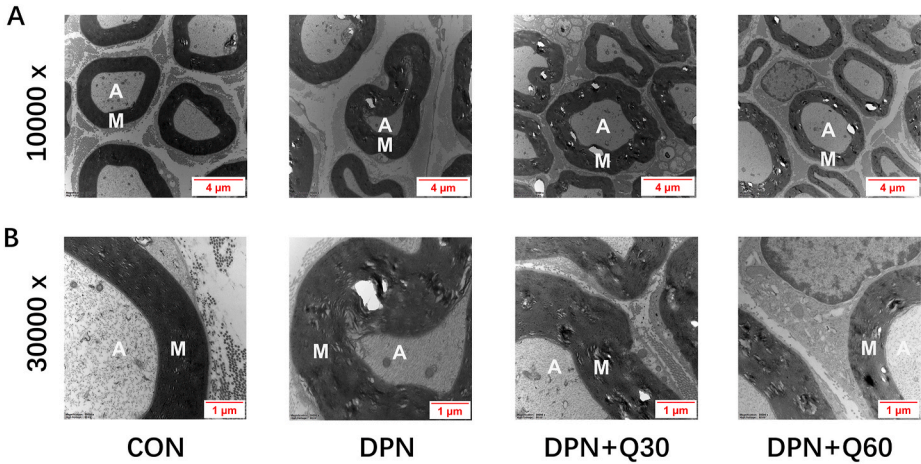


Fig. 5. Ultrastructural changes in sciatic nerves of DPN rats. The panel represents 10000× and 30000× magnified images (scale bar = 4 μm and 1 μm, respectively). The sciatic nerves of the CON group show myelinated fibres with regular round axons and an intact myelin sheath. The sciatic nerves of the DPN group show damaged myelin sheath with shrunken axons, vacuolisation, and lamellar separation. Quercetin administration (DPN + Q30 & DPN + Q60) ameliorates pathological changes in the sciatic nerve. A: axon; M: myelin; n = 3 per group. CON, control; DPN, diabetic peripheral neuropathy; Q30, quercetin at 30 mg/kg; Q60, quercetin at 60 mg/kg.

associated with DPN, the levels of TNF- α , IL-1 β , and IL-6 in the serum and sciatic nerves were determined using an ELISA kit. Compared with the CON group (Fig. 6A), the serum levels of TNF- α and IL-1 β were significantly elevated in the DPN group ($p < 0.001$). Conversely, quercetin (30 mg/kg and 60 mg/kg daily) significantly reduced TNF- α and IL-1 β expression levels ($p < 0.001$) when compared with the DPN group. In particular, serum levels of TNF- α and IL-1 β were lower in the DPN + Q60 group than in the DPN + Q30 group ($p < 0.001$). However, serum IL-6 levels were considerably low for detection, and repeated

measurements showed identical results.

In accordance with the above results, TNF- α and IL-1 β levels were higher in the sciatic nerves of the DPN group than those in the CON group ($p < 0.001$). Quercetin treatment significantly decreased the levels of TNF- α and IL-1 β in the sciatic nerves of DPN rats ($p < 0.05$ and $p < 0.001$, respectively). IL-6 levels were higher in the sciatic nerves of the DPN group than those in the CON group ($p < 0.001$). However, no differences in IL-6 levels were observed between the quercetin-treated and DPN model groups.

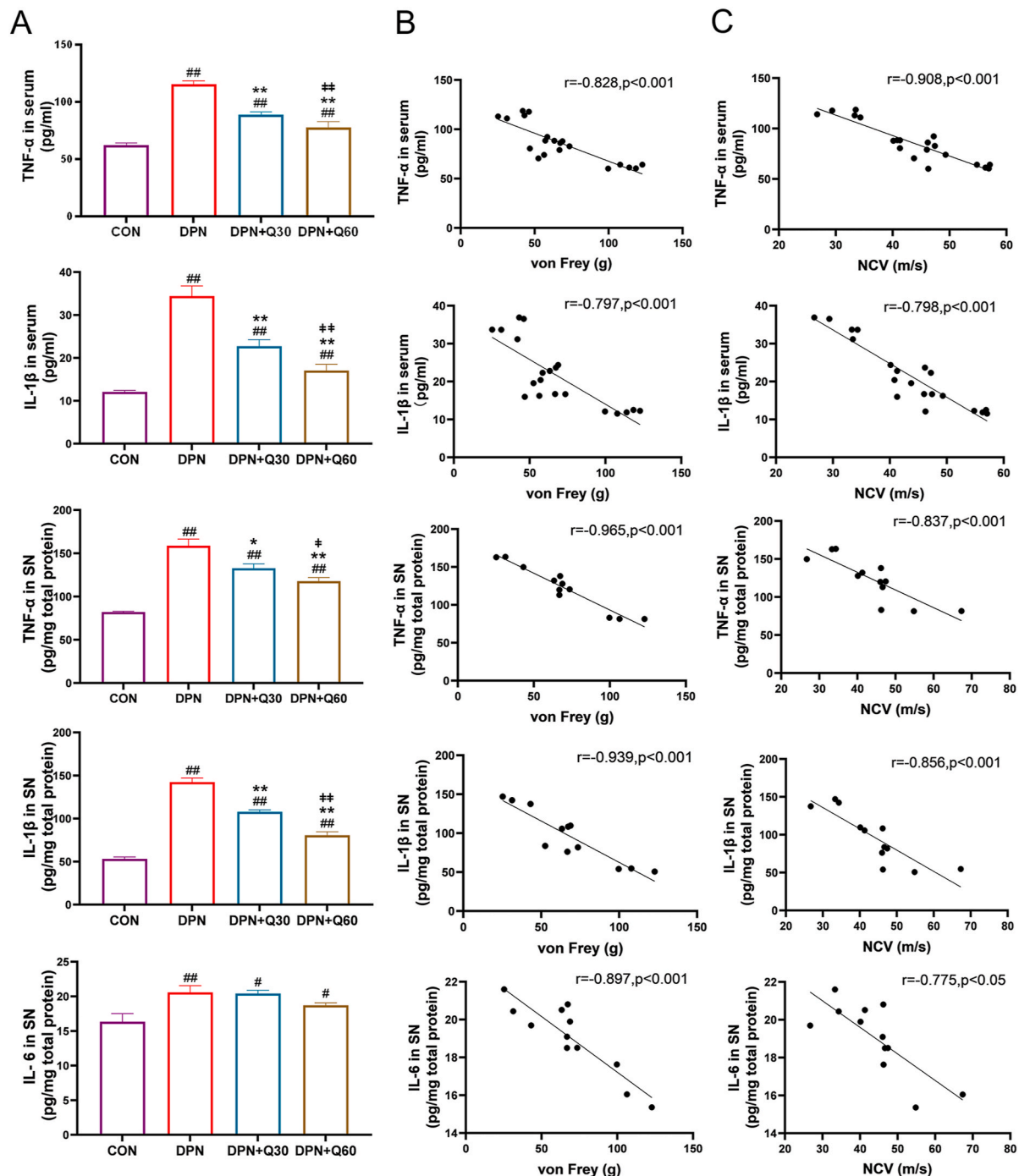


Fig. 6. Effect of quercetin on TNF- α , IL-1 β , IL-6 in sera and sciatic nerves and correlation analyses between inflammatory factors and DPN efficacy indicators. A: Inflammatory factors; B: von Frey test; C: Motor nerve conduction velocity (NCV). Data are expressed as mean $\pm$ standard deviation (SD) (sera, $n = 5$; sciatic nerves, $n = 3$ per group). ## $p < 0.001$, # $p < 0.05$ versus control rats; ** $p < 0.001$, * $p < 0.05$ versus DPN model rats; ## $p < 0.001$, # $p < 0.05$ versus DPN model rats treated with quercetin at 30 mg/kg by one-way ANOVA. CON, control; DPN, diabetic peripheral neuropathy; Q30, quercetin at 30 mg/kg; Q60, quercetin at 60 mg/kg; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6.

Moreover, correlation analyses were performed between inflammatory factors in the serum and sciatic nerves and DPN efficacy indicators (mechanical withdrawal threshold and NCV). As shown in Fig. 6B and C, TNF- α and IL-1 β in serum and TNF- α , IL-1 β , and IL-6 in the sciatic nerves negatively correlated with the mechanical withdrawal threshold ($r = -0.828, p < 0.001$; $r = -0.797, p < 0.001$; $r = -0.965, p < 0.001$; $r = -0.939, p < 0.001$; $r = -0.897, p < 0.001$, respectively). In addition, TNF- α and IL-1 β in the serum and TNF- α , IL-1 β , and IL-6 in sciatic nerves negatively correlated with NCV ($r = -0.908, p < 0.001$; $r = -0.798, p < 0.001$; $r = -0.837, p < 0.001$; $r = -0.856, p < 0.001$; $r = -0.775, p < 0.05$, respectively).

3.7. Quercetin inhibits the sciatic nerve expression of TLR4, MyD88, and p65 NF- κ B

Based on western blot analysis (Fig. 7), the levels of inflammatory proteins TLR4, MyD88, and p65 NF- κ B were markedly elevated in sciatic nerves of DPN rats when compared with CON rats ($p < 0.001$). In contrast, 30 mg/kg quercetin treatment downregulated the expression of MyD88 ($p < 0.001$) and p65 NF- κ B ($p < 0.05$) in DPN rats, but did not affect TLR4 ($p > 0.05$), when compared with DPN model rats. In particular, 60 mg/kg quercetin significantly decreased the expression of TLR4 ($p < 0.001$), MyD88 ($p < 0.001$), and p65 NF- κ B ($p < 0.001$) in the sciatic nerves of DPN rats. In addition, the levels of TLR4 ($p < 0.05$) and p65 NF- κ B ($p < 0.05$) were lower in the high-dose quercetin-treated group than those in the low-dose group.

3.8. IHC

Fig. 8 presents the immunohistochemical images of cross-sectional sciatic nerves. TLR4, MyD88, and NF- κ B were localised in Schwann cells in sciatic nerves. Consistent with the western blot results, immunohistochemical analysis of sciatic nerve tissues from DPN rats revealed abnormally elevated TLR4, MyD88, and p65 NF- κ B immunopositivity when compared with CON rats ($p < 0.001$). At 30 mg/kg, quercetin-treated DPN rats showed significantly reduced TLR4 ($p < 0.001$), MyD88 ($p < 0.001$), and p65 NF- κ B ($p < 0.001$) immunopositivity when compared with DPN rats. Furthermore, the expression of TLR4 ($p < 0.001$), MyD88 ($p < 0.001$), and p65 NF- κ B ($p < 0.001$) immunopositivity was considerably decreased in the sciatic nerves of DPN rats following treatment with 60 mg/kg quercetin when compared with those of untreated DPN rats. In addition, the expression levels of MyD88 ($p < 0.05$) and p65 NF- κ B ($p < 0.05$) were lower in the high-dose

quercetin-treated group than in the low-dose group.

4. Discussion

Accumulating evidence suggests that neurogenic inflammation contributes significantly to the pathogenesis of DPN (Stino et al., 2020; Elzinga et al., 2019). It has been reported that inflammatory cytokines, such as IL-1 β , are robustly associated with nerve injury, and TNF- α can result in oligodendrocyte toxicity and myelin damage (Shi et al., 2013b; Hangping et al., 2020). The flavonol quercetin is a naturally occurring polyphenol and reportedly possesses anti-inflammatory activity (Andres et al., 2018; Li et al., 2016). In the current study, quercetin administration did not affect the blood glucose levels and body weight of DPN rats. Some reports have suggested that quercetin could significantly ameliorate hyperglycemia in DM (Bule et al., 2019). Conversely, other studies have revealed that quercetin treatment failed to confer a notably favourable effect on blood glucose in DM (Xie et al., 2020). Based on previous results, we speculate that these discrepancies might arise due to the overall treatment time and dose range (Bhutada et al., 2010; Wang et al., 2012; Ebrahimpour et al., 2018). However, quercetin alleviated the pathological damage to sciatic nerves and DRG and partially recovered the peripheral neural function in DPN rats, including mechanical hyperalgesia and NCV without dose-relationship. In addition, quercetin reduced the levels of inflammatory factors TNF- α and IL-1 β in both the serum and sciatic nerves of DPN rats, indicating that quercetin may inhibit the inflammatory response under chronic hyperglycemic conditions. The underlying mechanism may be closely related to its regulatory effect on TLR4, MyD88, and NF- κ B, key proteins in TLR-associated pathways.

It has been well-established that the upregulation of TNF- α induces a series of inflammatory responses, which causes neuronal apoptosis and myelin damage to nerve fibres, leading to functional and structural deficits in the peripheral nervous system (Zhang et al., 2018; Shi et al., 2013a,b). In addition to TNF- α , some inflammatory factors such as IL-1 and IL-6 are involved in the onset and progression of DPN (Zheng et al., 2020; Nursal et al., 2019). Consistent with a previous report (Sacerdote et al., 2013), our results showed that the levels of TNF- α and IL-1 β were considerably higher in sciatic nerve tissues of DPN rats than in normal control rats. Meanwhile, serum levels of TNF- α and IL-1 β levels were significantly increased in DPN rats. According to the correlation analyses, these inflammatory factors, including TNF- α and IL-1 β , in the sciatic nerves both negatively correlated with functional indicators of peripheral nerves. These findings indicated that cytokine

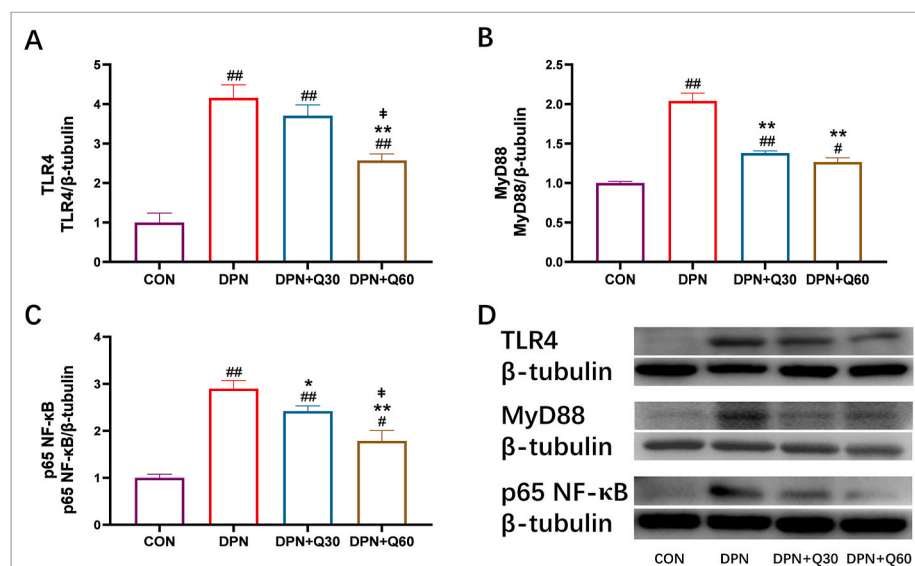


Fig. 7. Effect of quercetin on sciatic nerve expression of TLR4, MyD88, and p65 NF- κ B by western blotting. Data are expressed as mean $\pm$ standard deviation (SD) ($n = 3$ per group). ^{##} $p < 0.001$, [#] $p < 0.05$ versus control rats; ^{**} $p < 0.001$, ^{*} $p < 0.05$ versus DPN model rats; ^{##} $p < 0.001$, ^{*} $p < 0.05$ versus DPN model rats treated with quercetin at 30 mg/kg by one-way ANOVA. CON, control; DPN, diabetic peripheral neuropathy; Q30, quercetin at 30 mg/kg; Q60, quercetin at 60 mg/kg; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF- κ B, nuclear factor kappa B.

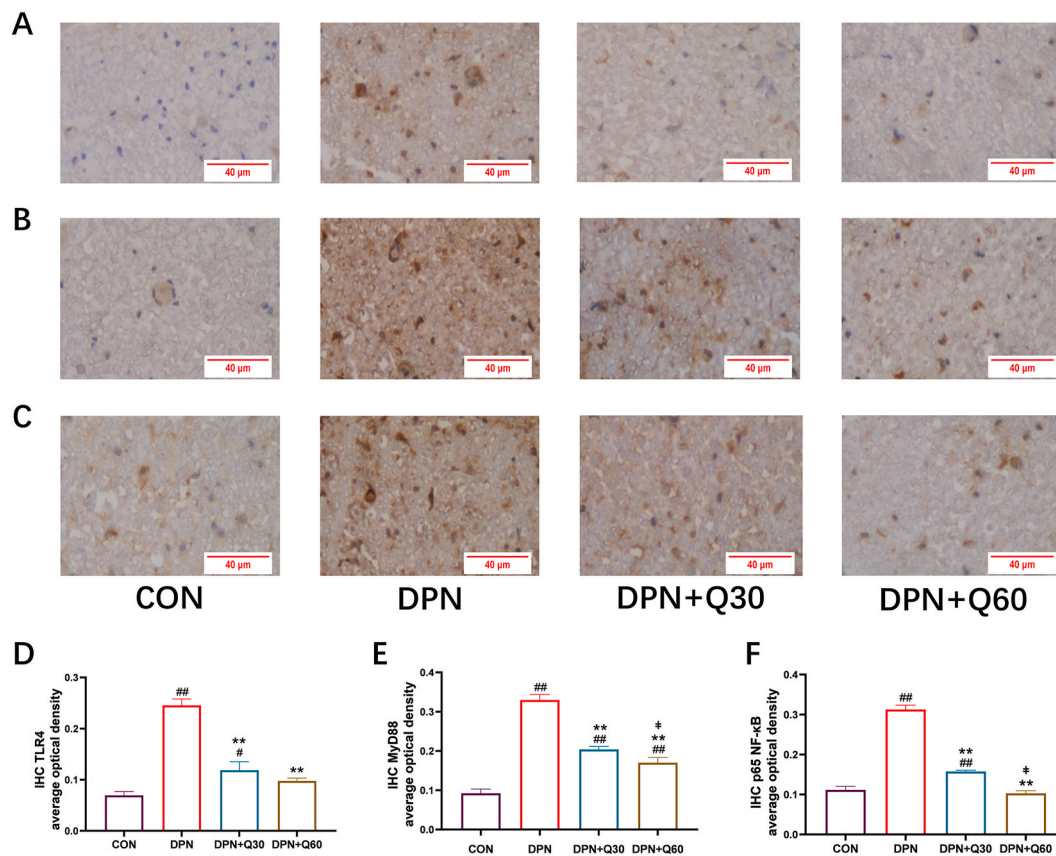


Fig. 8. Effect of quercetin on expression of TLR4, MyD88 and p65 NF-κB of sciatic nerves by immunohistochemistry. A: TLR4, B: MyD88, C: NF-κB. The panel represents 400 × magnified images (scale bar = 40 μm). Data are expressed as mean ± standard deviation (SD) (n = 3 per group). ##p < 0.001, #p < 0.05 versus control rats; **p < 0.001, *p < 0.05 versus DPN rats; ##p < 0.001, #p < 0.05 versus DPN model rats treated with quercetin at 30 mg/kg by one-way ANOVA. CON, control; DPN, diabetic peripheral neuropathy; Q30, quercetin at 30 mg/kg; Q60, quercetin at 60 mg/kg; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa B.

overexpression could promote sciatic nerve injury in DPN rats. It was reportedly that neuroprotective effect of quercetin on DPN rats might be partially associated with the downregulation of TNF-α and IL-1β levels in sciatic nerves (Kandhare et al., 2012). Consistent with previous investigation, our study revealed that quercetin administration significantly inhibited inflammatory factors in the serum and sciatic nerves of DPN rats. In addition, a previous study suggested that IL-6 levels were higher in the sera of DPN rats than those in the control group (Hasanvand et al., 2016). In contrast, another study has reported that serum IL-6 levels were lower in diabetic rats than in the control group (Dokumacioglu et al., 2018). In the present study, serum IL-6 levels could not be detected, suggesting that serum IL-6 levels were markedly low in rats. But IL-6 in the sciatic nerves was detected, and its level was low. The results showed that IL-6 levels in the sciatic nerve of DPN rats were significantly higher than those in the control group. Correlation analysis showed that IL-6 was negatively correlated with the functional indicators of peripheral nerves. But there was no statically significant difference in neural IL-6 between the quercetin-treated group and untreated DPN group. Based on these results, we hypothesised that IL-6 in the sciatic nerve might play a role in the development of DPN, and quercetin had no effect on the increased neural IL-6 levels. Axonal degeneration and myelin damage are the main pathological characteristics of DPN (Roman-Pintos et al., 2016). In the present study, quercetin treatment improved the typical pathological damage in the myelin sheath, axons, and neurons of DPN rats, as well as ameliorated neuropathic pain and NCV. These results revealed that quercetin had a neuroprotective effect, which was mainly related to the inhibition of the inflammatory response.

Furthermore, inflammatory factors such as TNF-α, IL-1, and IL-6,

which aggravate the inflammatory cascade reaction and cause damage to the peripheral nervous system, were modulated by TLR4-mediated signalling pathways. In mammals, 13 TLR members (TLR1-TLR13) have been identified in the TLR family, which recognise damage- and pathogen-associated molecular patterns (Kawai and Akira, 2007). TLR4 localises to the cell membrane and possesses a conserved cytoplasmic region that binds toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP) (Koushki et al., 2021; Chen et al., 2020). TLR4 can be activated by lipopolysaccharide or a variety of substances from damaged tissue, and the subsequent initiation of signal transduction triggers the production of inflammatory mediators, thereby eradicating pathogens (Litak et al., 2020; Lucas and Maes, 2013). A previous study has reported that TLR4 is highly expressed in the spinal cord and DRG of DPN rats (Jia et al., 2020; Chen et al., 2017). In addition, when TLR4 was inhibited in Schwann cells cultured in high glucose, inflammatory factors such as TNF-α and IL-1β were found to be markedly downregulated, and the cell survival ratio significantly increased (Tang et al., 2018b). In the present study, we observed that the expression of TLR4 in the sciatic nerves of DPN rats was significantly increased when compared with that in normal rats. Upregulation of TLR4 activates the downstream pathway, which promotes inflammatory cytokines to attack peripheral nerves (Tang et al., 2018a; Boivin et al., 2007). Moreover, we noted that quercetin significantly inhibited the expression of TLR4 in sciatic nerves of DPN rats, which could be beneficial for protecting peripheral nerves from injury.

TLR4-mediated functions are modulated via two different signalling pathways: the MyD88-dependent pathway and the MyD88-independent pathway (Rahimifard et al., 2017). In the present study, we observed that the expression of MyD88 was increased in sciatic nerves of DPN rats,

suggesting that MyD88 participates in the TLR4 mediated pathway during the progression of DPN. Notably, quercetin reversed elevated levels of TLR4. Following TLR4 activation, MyD88, as the cytoplasmic adapter protein, binds to the TLR4-TIRAP complex. Then, additional MyD88 proteins, IL-1 receptor-associated kinases (IRAKs), and TNF receptor-associated factor 6 (TRAF6) are recruited and jointly form a highly ordered structure termed the myddosome (Chen et al., 2020; Karimy et al., 2020). Myddosome formation activates transforming factor- β -activated kinase 1 (TAK1), leading to the dissociation of NF- κ B from the I κ B kinase complex (Anthoney et al., 2018; Karimy et al., 2020). As a result, this process leads to the activation of endonuclear signalling pathways, such as the transcription factor NF- κ B which promotes the transcription of inflammatory cytokines. The transcription factor NF- κ B family consists of NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), RelA (p65), RelB, and c-Rel, which exhibit different transcriptional functions. Among them, p65 NF- κ B is widely produced and has transcriptional activation domains that form dimers with other proteins of this family, thus modulating transcriptional activity (Rogero and Calder, 2018). After activation of I κ B kinase, I κ B protein is phosphorylated, ubiquitinated, and degraded by the proteasome to liberate the NF- κ B dimer in the cytoplasm (Williams and Gilmore, 2020). Then, the NF- κ B dimer is transferred to the nucleus and promotes the transcription activity of κ B-dependent genes, inducing the production of inflammatory factors such as TNF- α , IL-1 β , and IL-6 (Vincenzi et al., 2020). A previous study has demonstrated that NF- κ B significantly was increased in the lumbar spinal cord of DPN mice, along with inflammatory factors involved in diabetes-induced allodynia and hyperalgesia (Impellizzeri et al., 2019). It was reported that the mechanical withdrawal threshold and thermal withdrawal latency were increased in DPN rats following intrathecal injection of TLR4, MyD88, and NF- κ B antagonists. The inhibition of the TLR4/MyD88/NF- κ B pathway reduces inflammatory factors, contributing to the relief of diabetic neuropathic pain in DPN rats (Sun et al., 2019; Liu et al., 2018). In the present study, p65 NF- κ B was upregulated in the sciatic nerves of DPN rats, whereas quercetin inhibited the expression of p65 NF- κ B. The results were in accordance with the decreased TNF- α , IL-1 β , and IL-6 levels in sciatic nerves of quercetin-treated DPN rats.

Collectively, the present study indicates that neuroinflammation plays a pivotal role in the pathogenesis of DPN. Quercetin was found to reduce inflammatory factors such as TNF- α , IL-1 β , and IL-6, which could be related to the deterioration of nerve function. Moreover, quercetin could downregulate TLR4, MyD88, and p65 NF- κ B, the key proteins in the TLR4/MyD88/NF- κ B pathway, which may be associated with its neuroprotective and anti-inflammatory effects. However, there are some limitations of this study which should be addressed in future investigation. More additional biochemical experiments should be conducted to explore the relationship between quercetin and neuroinflammation based on high glucose-induced Schwann cells and neuronal damage. Meanwhile, the inflammatory mechanisms of DPN are complex. Therefore, it is necessary to further investigate the anti-inflammatory effects of quercetin on DPN through multiple inflammatory signalling pathways.

5. Conclusion

In summary, quercetin notably relieved neuropathic pain and improved NCV, as well as pathological changes in both sciatic nerves and DRG of STZ-induced diabetic rats. The neuroprotective effects of quercetin might be mediated by inhibiting inflammatory cytokines by downregulating the TLR4/MyD88/NF- κ B signalling pathway. Our study indicates that quercetin is a potential candidate for treating DPN.

Author contributions

XL contributed to the concept and design of the study and supervised this research. BZ and QZ analyzed the data and wrote the manuscript.

BZ, QZ, QS and JX performed the animal experiments. All authors have read and approved the final manuscript.

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CRediT authorship contribution statement

Bingjia Zhao: Investigation, Resources, Data curation, Writing – original draft. **Qian Zhang:** Investigation, Resources, Data curation, Writing – review & editing. **Xiaochun Liang:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, research. **Jun Xie:** Investigation. **Qing Sun:** Investigation.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

- Andres, Susanne, Pevny, Sophie, Ziegenhagen, Rainer, Bakhiya, Nadiya, Schäfer, Bernd, Hirsch-Ernst, Karen Ildico, Lampen, Alfonso, 2018. Safety aspects of the use of quercetin as a dietary supplement, 2018/01/01 Mol. Nutr. Food Res. 62 (1). <https://doi.org/10.1002/mnfr.201700447>. Accessed 2021/05/31, 1700447.
- Anjaneyulu, M., Chopra, K., 2004. Quercetin attenuates thermal hyperalgesia and cold allodynia in STZ-induced diabetic rats. *Indian J. Exp. Biol.* 42 (8), 766–769 (Aug).
- Anthoney, N., Foldi, I., Hidalgo, A., 2018. Toll and toll-like receptor signalling in development. *Development* 145 (9). <https://doi.org/10.1242/dev.156018> (Apr 25).
- Azam, S., Jakaria, M., Kim, I.S., Kim, J., Haque, M.E., Choi, D.K., 2019. Regulation of toll-like receptor (Tlr) signaling pathway by polyphenols in the treatment of age-linked neurodegenerative diseases: focus on Tlr4 signaling. *Front. Immunol.* 10, 1000. <https://doi.org/10.3389/fimmu.2019.01000>.
- Bhutada, P., Mundhada, Y., Bansod, K., Bhutada, C., Tawari, S., Dixit, P., Mundhada, D., 2010. Ameliorative effect of quercetin on memory dysfunction in streptozotocin-induced diabetic rats. *Neurobiol. Learn. Mem.* 94 (3), 293–302. <https://doi.org/10.1016/j.nlm.2010.06.008> (Oct).
- Biessels, G.J., Bril, V., Calcutt, N.A., Cameron, N.E., Cotter, M.A., Dobrowsky, R., Feldman, E.L., Fernyhough, P., Jakobsen, J., Malik, R.A., Mizisin, A.P., Oates, P.J., Obrosova, I.G., Pop-Busui, R., Russell, J.W., Sima, A.A., Stevens, M.J., Schmidt, R.E., Tesfaye, S., Veves, A., Vinik, A.I., Wright, D.E., Yagihashi, S., Yorek, M.A., Ziegler, D., Zochodne, D.W., 2014. Phenotyping animal models of diabetic neuropathy: a consensus statement of the diabetic neuropathy study group of the Easd (Neurodiab). *J. Peripher. Nerv. Syst.* 19 (2), 77–87. <https://doi.org/10.1111/jns5.12072> (Jun).
- Boivin, Audrey, Pineau, Isabelle, Benoit, Barrette, Filali, Mohammed, Vallières, Nicolas, Rivest, Serge, Steve Lacroix, 2007. Toll-like receptor signaling is critical for wallerian degeneration and functional recovery after peripheral nerve injury. *J. Neurosci. : Off. J. Soc. Neurosci.* 27 (46), 12565–12576. <https://doi.org/10.1523/JNEUROSCI.3027-07.2007>.
- Bule, M., Abdurahman, A., Nikfar, S., Abdollahi, M., Amini, M., 2019. Antidiabetic effect of quercetin: a systematic review and meta-analysis of animal studies. *Food Chem. Toxicol.* 125, 494–502. <https://doi.org/10.1016/j.fct.2019.01.037> (Mar).
- Calvo-Rodriguez, M., Garcia-Rodriguez, C., Villalobos, C., Nunez, L., 2020. Role of toll like receptor 4 in alzheimer's disease. *Front. Immunol.* 11, 1588. <https://doi.org/10.3389/fimmu.2020.01588>.
- Chen, L., Zheng, L., Chen, P., Liang, G., 2020. Myeloid differentiation primary response protein 88 (Myd88): the central hub of Tlr/Il-1r signaling. *J. Med. Chem.* 63 (22), 13316–13329. <https://doi.org/10.1021/acs.jmedchem.0c00884> (Nov 25).
- Chen, T., Li, H., Yin, Y., Zhang, Y., Liu, Z., Liu, H., 2017. Interactions of Notch1 and Tlr4 signaling pathways in Drg neurons of in vivo and in vitro models of diabetic neuropathy. *Sci. Rep.* 7 (1) <https://doi.org/10.1038/s41598-017-15053-w> (Nov 2), 14923.

- Dokumacioglu, E., Iskender, H., Sen, T.M., Ince, I., Dokumacioglu, A., Kanbay, Y., Erbas, E., Saral, S., 2018. The effects of hesperidin and quercetin on serum tumor necrosis factor- α and interleukin-6 levels in streptozotocin-induced diabetes model. *Pharmacogn Mag* 14 (54), 167–173. https://doi.org/10.4103/pm_pm_41_17 (Apr-Jun).
- Ebrahimpour, S., Esmaeili, A., Beheshti, S., 2018. Effect of quercetin-conjugated superparamagnetic iron oxide nanoparticles on diabetes-induced learning and memory impairment in rats. *Int. J. Nanomed.* 13, 6311–6324. <https://doi.org/10.2147/ijn.s177871>.
- Elzinga, S., Murdock, B.J., Guo, K., Hayes, J.M., Tabbey, M.A., Hur, J., Feldman, E.L., 2019. Toll-like receptors and inflammation in metabolic neuropathy: a role in early versus late disease? *Exp. Neurol.* 320, 112967. <https://doi.org/10.1016/j.expneurol.2019.112967> (Oct).
- Feldman, E.L., Callaghan, B.C., Pop-Busui, R., Zochodne, D.W., Wright, D.E., Bennett, D. L., Bril, V., Russell, J.W., Viswanathan, V., 2019. Diabetic neuropathy. *Nat Rev Dis Primers* 5 (1), 41. <https://doi.org/10.1038/s41572-019-0092-1> (Jun 13).
- Furman, B.L., 2015. Streptozotocin-induced diabetic models in mice and rats. *Curr Protoc Pharmacol* 70. <https://doi.org/10.1002/0471141755.ph0547s70> (Sep 1): 5 47 1-5 47 20.
- Hangping, Z., Ling, H., Lijin, J., Wenting, Z., Xiaoxia, L., Qi, Z., Xiaoming, Z., Qingchun, L., Yiming, L., Qian, X., Ji, H., Bin, L., Shuo, Z., 2020. The preventive effect of IL-1 β antagonist on diabetic peripheral neuropathy. *Endocr. Metab. Immune Disord. - Drug Targets* 20 (5), 753–759. <https://doi.org/10.2174/1871530319666191022114139>.
- Hasanvand, A., Amini-Khoei, H., Hadian, M.R., Abdollahi, A., Tavangar, S.M., Dehpour, A.R., Semiei, E., Mehr, S.E., 2016. Anti-inflammatory effect of ampk signaling pathway in rat model of diabetic neuropathy. *Inflammopharmacology* 24 (5), 207–219. <https://doi.org/10.1007/s10787-016-0275-2> (Oct).
- Impellizzeri, D., Peritore, A.F., Cordaro, M., Gugliandolo, E., Siracusa, R., Crupi, R., D'Amico, R., Fusco, R., Evangelista, M., Cuzzocrea, S., Di Paola, R., 2019. The neuroprotective effects of micronized pea (Pea-M) formulation on diabetic peripheral neuropathy in mice. *Faseb. J.* 33 (10), 11364–11380. <https://doi.org/10.1096/fj.201900538R> (Oct).
- Jia, G.L., Huang, Q., Cao, Y.N., Xie, C.S., Shen, Y.J., Chen, J.L., Lu, J.H., Zhang, M.B., Li, J., Tao, Y.X., Cao, H., 2020. Cav-1 participates in the development of diabetic neuropathy pain through the Tlr4 signaling pathway. *J. Cell. Physiol.* 235 (3). <https://doi.org/10.1002/jcp.29106> (Mar): 2060–2070.
- Kandhare, Amit D., Raygude, Kiran S., Kumar, V., Shiva, Rajmane, Anuchadra R., Visnagri, Asjad, Ghule, Arvindkumar E., Ghosh, Pinaki, Badole, Sachin L., Bodhankar, Subhash L., 2012. Ameliorative effects quercetin against impaired motor nerve function, inflammatory mediators and apoptosis in neonatal streptozotocin-induced diabetic neuropathy in rats. *Biomed. Aging Pathol.* 2 (4), 173–186. <https://doi.org/10.1016/j.biomag.2012.10.002>.
- Karim, J.K., Reeves, B.C., Kahle, K.T., 2020. Targeting Tlr4-dependent inflammation in post-hemorrhagic brain injury. *Expert Opin. Ther. Targets* 24 (6), 525–533. <https://doi.org/10.1080/14728222.2020.1752182> (Jun).
- Kawai, T., Akira, S., 2007. Tlr signaling. *Semin. Immunol.* 19 (1), 24–32. <https://doi.org/10.1016/j.smim.2006.12.004> (Feb).
- Koushki, K., Shahbaz, S.K., Mashayekhi, K., Sadeghi, M., Zayeri, Z.D., Taba, M.Y., Banach, M., Al-Rasadi, K., Johnston, T.P., Sahebkar, A., 2021. Anti-inflammatory action of statins in cardiovascular disease: the role of inflammasome and toll-like receptor pathways. *Clin. Rev. Allergy Immunol.* 60 (2), 175–199. <https://doi.org/10.1007/s12016-020-08791-9> (Apr).
- Lees, J.G., Makker, P.G., Tonkin, R.S., Abdulla, M., Park, S.B., Goldstein, D., Moalem-Taylor, G., 2017. Immune-mediated processes implicated in chemotherapy-induced peripheral neuropathy. *Eur. J. Cancer* 73, 22–29. <https://doi.org/10.1016/j.ejca.2016.12.006> (Mar).
- Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M.T., Wang, S., Liu, H., Yin, Y., 2016. Quercetin, inflammation and immunity. *Nutrients* 8 (3), 167. <https://doi.org/10.3390/nu8030167> (Mar 15).
- Litak, J., Grochowski, C., Litak, J., Osuchowska, I., Gosik, K., Radzikowska, E., Kamieniak, P., Rolinski, J., 2020. Tlr-4 signaling vs. Immune checkpoints, mirnas molecules, cancer stem cells, and wntless-signaling interplay in glioblastoma multiforme-future perspectives. *Int. J. Mol. Sci.* 21 (9). <https://doi.org/10.3390/ijms21093114> (Apr 28).
- Liu, P., Yuan, H.B., Zhao, S., Liu, F.F., Jiang, Y.Q., Guo, Y.X., Wang, X.L., 2018. Activation of gabab receptor suppresses diabetic neuropathic pain through toll-like receptor 4 signaling pathway in the spinal dorsal horn. *Mediat. Inflamm.* 6016272. <https://doi.org/10.1155/2018/6016272>, 2018.
- Lucas, K., Maes, M., 2013. Role of the toll like receptor (Tlr) radical cycle in chronic inflammation: possible treatments targeting the Tlr4 pathway. *Mol. Neurobiol.* 48 (1), 190–204. <https://doi.org/10.1007/s12035-013-8425-7> (Aug).
- Mu, Z.P., Wang, Y.G., Li, C.Q., Lv, W.S., Wang, B., Jing, Z.H., Song, X.J., Lun, Y., Qiu, M. Y., Ma, X.L., 2017. Association between tumor necrosis factor- α and diabetic peripheral neuropathy in patients with type 2 diabetes: a meta-analysis. *Mol. Neurobiol.* 54 (2), 983–996. <https://doi.org/10.1007/s12035-016-9702-z> (Mar).
- Nursal, A.F., Inanir, A., Rustemoglu, A., Uzun, S., Sahin, K., Yigit, S., 2019. Il-1 β and Il-1ra variant profiles in Turkish patients with diabetic peripheral neuropathy. *Endocr. Metab. Immune Disord. - Drug Targets* 19 (2), 150–158. <https://doi.org/10.2174/1871530318666181022165048>.
- Rahimifard, M., Maqbool, F., Moeini-Nodeh, S., Niaz, K., Abdollahi, M., Braid, N., Nabavi, S.M., Nabavi, S.F., 2017. Targeting the Tlr4 signaling pathway by polyphenols: a novel therapeutic strategy for neuroinflammation. *Ageing Res. Rev.* 36, 11–19. <https://doi.org/10.1016/j.arr.2017.02.004> (Jul).
- Rogero, M.M., Calder, P.C., 2018. Obesity, inflammation, toll-like receptor 4 and fatty acids. *Nutrients* 10 (4). <https://doi.org/10.3390/nu10040432> (Mar 30).
- Roman-Pintos, L.M., Villegas-Rivera, G., Rodríguez-Carrizalez, A.D., Miranda-Díaz, A.G., Cardona-Munoz, E.G., 2016. Diabetic polyneuropathy in type 2 diabetes mellitus: inflammation, oxidative stress, and mitochondrial function. *J. Diabetes Res* 3425617. <https://doi.org/10.1155/2016/3425617>, 2016.
- Sacerdote, P., Franchi, S., Moretti, S., Castelli, M., Procacci, P., Magnaghi, V., Panerai, A. E., 2013. Cytokine modulation is necessary for efficacious treatment of experimental neuropathic pain. *J. Neuroimmune Pharmacol.* 8 (1), 202–211. <https://doi.org/10.1007/s11481-012-9428-2> (Mar).
- 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., 2019. Temporary removal: global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: results from the international diabetes federation diabetes Atlas, 9 (Th) edition. *Diabetes Res Clin Pract* 107843. <https://doi.org/10.1016/j.diabres.2019.107843> (Sep 10).
- Sandireddy, Reddemma, Yerra, Veera Ganesh, Areti, Aparna, Komirishetty, Prashanth, Kumar, Ashutosh, 2014. Neuroinflammation and oxidative stress in diabetic neuropathy: futuristic strategies based on these targets. *Int. J. Endocrinol.* 1–10. <https://doi.org/10.1155/2014/674987>, 2014.
- Schlesinger, S., Herder, C., Kannenberg, J.M., Huth, C., Carstensen-Kirberg, M., Rathmann, W., Bonhof, G.J., Koenig, W., Heier, M., Peters, A., Meisinger, C., Roden, M., Thorand, B., Ziegler, D., 2019. General and abdominal obesity and incident distal sensorimotor polyneuropathy: insights into inflammatory biomarkers as potential mediators in the Kora F4/Ff4 cohort. *Diabetes Care* 42 (2), 240–247. <https://doi.org/10.2337/dc18-1842> (Feb).
- Shi, G.J., Li, Y., Cao, Q.H., Wu, H.X., Tang, X.Y., Gao, X.H., Yu, J.Q., Chen, Z., Yang, Y., 2019. In vitro and in vivo evidence that quercetin protects against diabetes and its complications: a systematic review of the literature. *Biomed. Pharmacother.* 109, 1085–1099. <https://doi.org/10.1016/j.biopha.2018.10.130> (Jan).
- Shi, X., Chen, Y., Nadeem, L., Xu, G., 2013a. Beneficial effect of Tnf- α inhibition on diabetic peripheral neuropathy. *J. Neuroinflammation* 10, 69. <https://doi.org/10.1186/1742-2094-10-69> (Jun 4).
- Shi, Xiaohong, Chen, Yinghui, Nadeem, Lubna, Xu, Guoxiong, 2013b. Beneficial effect of Tnf- α inhibition on diabetic peripheral neuropathy. *J. Neuroinflammation* 10. <https://doi.org/10.1186/1742-2094-10-69>, 69–69.
- Spagnuolo, C., Moccia, S., Russo, G.L., 2018. Anti-inflammatory effects of flavonoids in neurodegenerative disorders. *Eur. J. Med. Chem.* 153 (Jun 10), 105–115. <https://doi.org/10.1016/j.ejmech.2017.09.001>.
- Stino, A.M., Rumora, A.E., Kim, B., Feldman, E.L., 2020. Evolving concepts on the role of dyslipidemia, bioenergetics, and inflammation in the pathogenesis and treatment of diabetic peripheral neuropathy. *J. Peripher. Nerv. Syst.* 25 (2), 76–84. <https://doi.org/10.1111/jns.12387> (Jun).
- Sun, Q., Zhang, B.Y., Zhang, P.A., Hu, J., Zhang, H.H., Xu, G.Y., 2019. Downregulation of glucose-6-phosphate dehydrogenase contributes to diabetic neuropathic pain through upregulation of toll-like receptor 4 in rats. *Mol. Pain* 15. <https://doi.org/10.1177/1744806919838659> (Jan-Dec): 1744806919838659.
- Tang, G., Yao, J., Shen, R., Ji, A., Ma, K., Cong, B., Wang, F., Zhu, L., Wang, X., Ding, Y., Zhang, B., 2018a. Reduced inflammatory factor expression facilitates recovery after sciatic nerve injury in Tlr4 mutant mice. *Int. Immunopharm.* 55, 77–85. <https://doi.org/10.1016/j.intimp.2017.12.007>.
- Tang, W., Chen, X., Liu, H., Lv, Q., Zou, J., Shi, Y., Liu, Z., 2018b. Expression of Nrf2 promotes Schwann cell-mediated sciatic nerve recovery in diabetic peripheral neuropathy. *Cell. Physiol. Biochem.* 46 (5), 1879–1894. <https://doi.org/10.1159/000489373>.
- Vincenzi, A., Goettert, M.I., Volken de Souza, C.F., 2020. An evaluation of the effects of probiotics on tumoral necrosis factor (Tnf- α) signaling and gene expression. *Cytokine Growth Factor Rev.* <https://doi.org/10.1016/j.cytogr.2020.10.004> (Nov 2).
- Wang, C., Pan, Y., Zhang, Q.Y., Wang, F.M., Kong, L.D., 2012. Quercetin and allopurinol ameliorate kidney injury in Stz-treated rats with regulation of renal Nlrp3 inflammasome activation and lipid accumulation. *PLoS One* 7 (6), e38285. <https://doi.org/10.1371/journal.pone.0038285>.
- Williams, Leah M., Gilmore, Thomas D., 2020. Looking down on Nf-Kb. *Mol. Cell Biol.* 40 (15), e00104–e00120. <https://doi.org/10.1128/MCB.00104-20>.
- Wu, Meiyang, Liu, Fengting, Guo, Qinghui, 2019. Quercetin attenuates hypoxia-ischemia induced brain injury in neonatal rats by inhibiting Tlr4/Nf-Kb signaling pathway. *Int. Immunopharm.* 74, 105704. <https://doi.org/10.1016/j.intimp.2019.105704> (2019/09/01/).
- Xie, J., Song, W., Liang, X., Zhang, Q., Shi, Y., Liu, W., Shi, X., 2020. Protective effect of quercetin on streptozotocin-induced diabetic peripheral neuropathy rats through modulating gut microbiota and reactive oxygen species level. *Biomed. Pharmacother.* 127, 110147. <https://doi.org/10.1016/j.biopha.2020.110147> (Jul).
- Yang, R., Li, L., Yuan, H., Liu, H., Gong, Y., Zou, L., Li, S., Wang, Z., Shi, L., Jia, T., Zhao, S., Wu, B., Yi, Z., Gao, Y., Li, G., Xu, H., Liu, S., Zhang, C., Li, G., Liang, S., 2019. Quercetin relieved diabetic neuropathic pain by inhibiting upregulated P2x4 receptor in dorsal root ganglia. *J. Cell. Physiol.* 234 (3), 2756–2764. <https://doi.org/10.1002/jcp.27091> (Mar).
- Zhang, K., Yang, S., Luo, C., 2018. Tnf- α and Tnf-R1 regulate bupivacaine-induced apoptosis in cord dorsal root ganglion neuron. *Eur. J. Pharmacol.* 833, 63–68. <https://doi.org/10.1016/j.ejphar.2018.05.034>.
- Zhang, Q., Song, W., Liang, X., Xie, J., Shi, Y., Shi, X., Qiu, B., Chen, X., 2020. A metabolic insight into the neuroprotective effect of Jin-Mai-Tong (Jmt) decoction on diabetic rats with peripheral neuropathy using untargeted metabolomics strategy. *Front. Pharmacol.* 11, 221. <https://doi.org/10.3389/fphar.2020.00221>.
- Zheng, Hangping, Sun, Wanwan, Zhang, Qi, Zhang, Yuanpin, Ji, Lijin, Liu, Xiaoxia, Zhu, Xiaoming, Ye, Hongying, Xiong, Qian, Li, Yiming, Lu, Bin, Zhang, Shuo, 2020. Proinflammatory cytokines predict the incidence of diabetic peripheral neuropathy

over 5 Years in Chinese type 2 diabetes patients: a prospective cohort study. EClin. Med. 31 <https://doi.org/10.1016/j.eclinm.2020.100649>, 100649-100649.