Drug Design, Development and Therapy

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Network Pharmacology Combined With Metabolomics Reveals the Mechanism of Yangxuerongjin Pill Against Type 2 Diabetic Peripheral Neuropathy in Rats

Ran Jin*, Hailuan Pei*, Feng Yue, Xiaodi Zhang, Zhicong Zhang, Yi Xu, Jinsheng Li

Beijing Tongrentang Technology Development Co., Ltd. Pharmaceutical Factory, Beijing, 100079, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yi Xu; Jinsheng Li, Beijing Tongrentang Technology Development Co., Ltd. Pharmaceutical factory, Beijing, 100079, People's Republic of China, Tel +86-10-87632655, Email xy706@sina.com; yarily@126.com

Purpose: This study aims to explore the mechanism of Yangxuerongjin pill (YXRJP) in the treatment of diabetic peripheral neuropathy (DPN) by network pharmacology and metabolomics technology combined with animal experiments, and to provide scientific basis for the treatment of DPN.

Methods: In this study, network pharmacology analysis was applied to identify the active compounds, core targets and signal pathways, which might be responsible for the effect of DPN. The DPN model was established by high-fat diet combined with streptozotocin (STZ) injection, and the rats were given administration for 12 weeks. The body weight, thermal withdrawal latency (TWL), sciatic motor nerve conduction velocity (MNCV), biochemical indexes, pathological sections of sciatic nerve, oxidative stress factors and the expression levels of neuroprotection-related proteins were detected. Metabolomics technology was used to analyze the potential biomarkers and potential metabolic pathways in DPN treated with YXRJP.

Results: The results of network pharmacology showed that YXRJP could treat DPN through baicalin, β-sitosterol, 7-methoxy-2-methylisoflavone, aloe-emodin and luteolin on insulin resistance, Toll-like receptor (TLR), tumor necrosis factor (TNF) and other signaling pathways. YXRJP can prolong the TWL, increase the MNCV of the sciatic nerve, alleviate the injury of the sciatic nerve, reduce the levels of triglyceride (TG), improve the expression of Insulin-like growth factor 1 (IGF-1) protein in the sciatic nerve, and reduce the expression of protein kinase B (AKT) protein. Metabolomics results showed that the potential metabolic pathways of YXRJP in the treatment of DPN mainly involved amino acid metabolism such as arginine, alanine, aspartic acid, lipid metabolism and nucleotide metabolism.

Conclusion: YXRJP can effectively improve the symptoms of DPN rats and reduce nerve damage. The effects are mainly related to reducing oxidative stress injury, promoting the expression of neuroprotection-related proteins, reducing the expression of inflammation-related proteins, and affecting amino acid metabolism, lipid metabolism, and nucleotide metabolism pathways. Our findings revealed that YXRJP has a good therapeutic potential for DPN, which provides a reference for further studies on YXRJP.

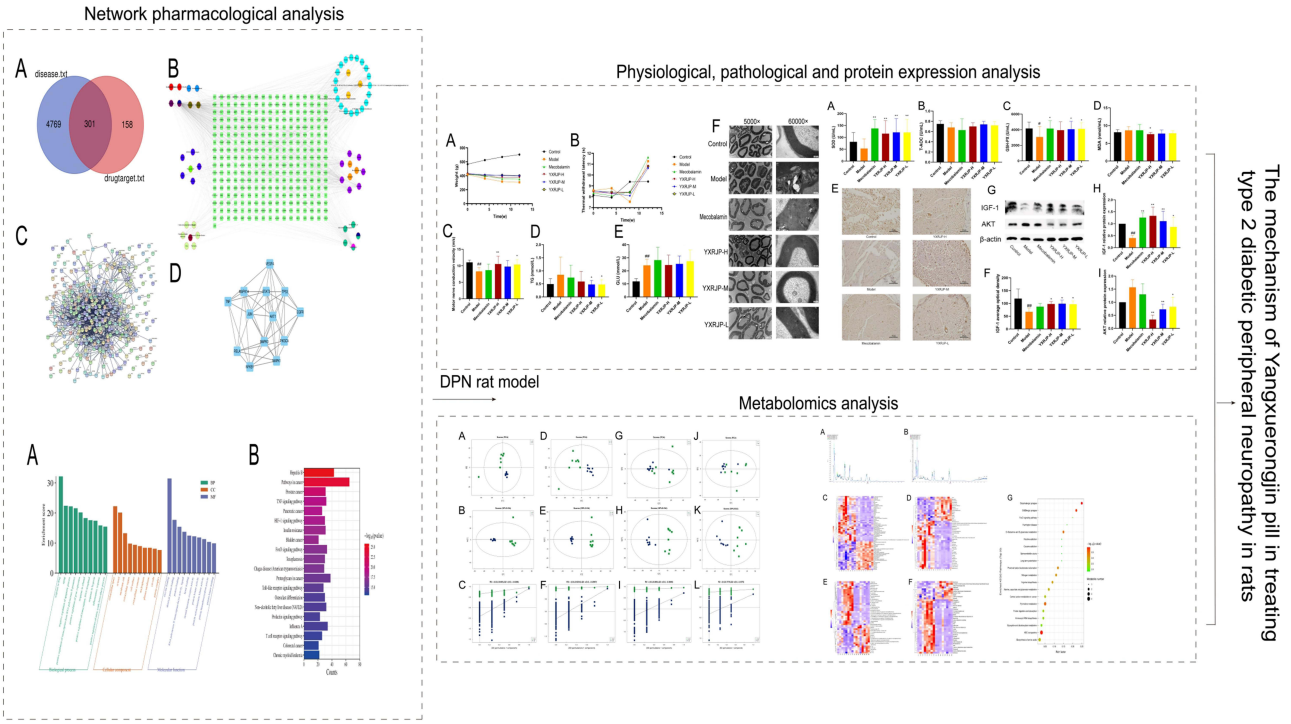
Keywords: Yangxuerongjin pill, Type 2 Diabetes, Rat, peripheral neuropathy, network pharmacology, metabolomics

Introduction

Diabetic peripheral neuropathy (DPN) is one of the complications of type 2 diabetes, and due to its complex pathogenesis and high incidence, it has become one of the most valued common complications of chronic diabetes in the world.[1] The International Diabetes Federation (IDF) reports, in 2021, there were 537 million people with diabetes in the world, with a prevalence of about 10.5% of the world's adult population. By 2045, the absolute number of people with diabetes is expected to increase by 46%, with 783 million people living with diabetes globally.[2] DPN is one of the most common

Received: 20 May 2024
Accepted: 28 December 2024
Published: 16 January 2025

Graphical Abstract



complications in diabetic patients. The global prevalence of DPN in diabetic patients has reached over 50%, which is still increasing.[3] At present, diabetes is still one of the leading causes of death in the world, ranking in the top 10.[4] The World Health Organization (WHO) predicts that complications from this condition are expected to be the primary cause of mortality by 2030.[5] The body of patients with DPN is in a state of high glucose (GLU), which promotes the glycosylation of neuromyelin proteins, and the glycosylated myelin proteins are specifically recognised and phagocytosed by macrophages, which leads to the loss of demyelination in nerve cells and abnormalities in peripheral nerve conduction.[6] Clinically, the symptoms of DPN are insidious and easy to be ignored by the patients. With the gradual aggravation of the disease, it manifests itself as symmetrical limb sensory and motor abnormalities, such as numbness and tingling of the limbs, etc., and develops into more extreme neuropathic pain in about 10–30% of the patients. With loss of sensation, limitation of movement, and atrophy of the muscle groups in the later stage, and the development of the disease may ultimately lead to amputation. It is one of the most important causes of disability and death in diabetic patients.[7–9] Currently there is no specific treatment plan and recommended therapeutic drugs, mainly through the improvement of blood GLU levels and the corresponding symptomatic treatment to prevent and alleviate DPN, but there are serious side effects and other problems,[10] so it is urgent to find effective and safe treatment drugs. With the deepening of the research on chronic diseases and metabolic diseases in traditional Chinese medicine (TCM), the effect of TCM on the prevention and treatment of DPN has gradually attracted the attention of clinical workers.

DPN can be classified as “thirst paralysis” in TCM, which is caused by prolonged thirst, depletion of Qi and injury of fluids, resulting in deficiency of Qi and blood, internal obstruction of blood stasis, loss of moistening of tendons and veins and lack of glorification, which leads to the disease.[11] According to Su Wen Miao Thoracic Lecture, it is put under the category of “Collaterals Disease”, which is related to prolonged entry into the collaterals. The disease mechanism can be divided into stagnation leading to pain and nourishment leading to pain. When thirst persists for a long time, phlegm, dampness, stasis and other solid pathogenic factors stagnate in the collaterals. Once the collaterals are obstructed by stagnation, symptoms such as numbness and pain in the limbs may appear. In this case, the treatment method of

activating blood circulation, removing phlegm and dredging collaterals can be employed. When thirst is prolonged, deficiency of Qi and blood, loss of nourishment of channels and collaterals, and lack of nourishment, there are signs of fatigue, pain, decrease in temperature of limbs, and dryness of skin, etc., which should be treated by tonifying Qi, nourishing blood and dredging collaterals.[12]

The original recipe of Yangxuerongjin pill (YXRJP) comes from Xingyuanshengchun of the Ming Dynasty. It has the functions of nourishing blood and nourishing sinew, dispelling wind and dredging collaterines. YXRJP is composed of 16 Chinese herbs, such as *Angelica sinensis* (Oliv). Diels, *Spatholobus suberectus* Dunn, *Pleuropterus multiflorus* (Thunb). Turcz. ex Nakai (stir-frying with wine and black soya bean), *Paeonia anomala* L., *Dipsacus asper* Wall. ex C.B. Clarke, *Taxillus sutchuenensis* (Lecomte) Danser, *Clematis chinensis* Osbeck (stir-frying with wine), *Lycopodium japonicum* Thunb., *Phryma leptostachya* L., *Pinus massoniana* Lamb., *Psoralea corylifolia* L. (stir-frying with salt), *Codonopsis pilosula* (Franch). Nannf., *Atractylodes macrocephala* Koidz., *Citrus reticulata* Blanco, *Aucklandia lappa* Decne., *Vigna umbellata* (Thunb). Ohwi & H.Ohashi, the ratio is: 3:5:10:5:5:5:3:5:3:3:4:5:4:3:3:5,[13] the plant name have been checked with http://www.theplantlist.org. The combination of all the herbs regulates Qi and blood and nourishes liver and kidneys together to treat the root cause. Dispelling wind and dehumidification, promoting blood circulation, dredging collaterals and relieving pain can be used to treat its symptoms. It is an effective remedy for old fall injury, muscle and bone pain, limb numbness, muscle atrophy, and joint stiffness. Studies have shown that YXRJP can treat various kinds of nerve pain and nerve compression numbness, such as sciatic nerve pain, intercostal neuralgia and occipital neuralgia.[14] YXRJP combined with acupuncture can improve the incoordination and even disorder of muscle group movement caused by peripheral nervous system abnormalities in stroke patients.[15] At the same time, the components of YXRJP also have good therapeutic effect on DPN, which can reduce nerve damage.[16–20] The active components of YXRJP, such as naringenin, ellagic acid, baicalin, baicalein, luteolin, quercetin and kaempferol, have been confirmed to have neuroprotective effects.[21–27] Our previous experimental study found that YXRJP had a good neuroprotective effect on sciatic nerve injury caused by clamping and peripheral neurotoxicity induced by chemotherapy.[28] In order to further explore the preventive and therapeutic effects of drugs on peripheral nerve injury and its mechanism, the active ingredients and action targets of YXRJP were predicted by network pharmacology. We took mecobalamin, a neurotrophic drug with clear clinical efficacy, as the positive control group.[29–31] This can verify the reliability of the experimental method and provide a reference standard for the evaluation of the experimental drug's effect, thus helping the experimenter better judge the action characteristics of the experimental drug. The efficacy of YXRJP was evaluated by observing clinical indications (MNCV, pain abnormality and histopathological changes) in type 2 diabetic rats. By detecting factors associated with the pathogenesis of DPN (such as oxidative stress factors, inflammatory factors, neurotrophic factors, etc)., and combining network pharmacology and metabolomics technologies, we preliminarily explore the mechanism of YXRJP treatment for DPN. This study provides pharmacological experimental evidence for fully developing the clinical value of YXRJP and finding effective drugs for clinical treatment of DPN.

Materials and Methods

Drugs and Reagents

Yangxuerongjin pill was purchased from Beijing Tongrentang Technology Development Co., Ltd. Pharmaceutical Factory (batch number: 20010356). Mecobalamin tablets was purchased from Eisai Pharmaceutical Co., Ltd (batch number: 2004074). Special feed (45 kcal% fat calorie high Fat food) was purchased from Deitz Biotechnology Co., Ltd (batch number: 102629). Streptozotocin (STZ) was purchased from Sigma Company (batch number: WXBD2372V). Insulin-like growth factor 1 (IGF-1) was purchased from Bioworld Technology (batch number: CC02181). The GLU kit and triglyceride (TG) kit were purchased from BioSino Bio-Technology Science Inc. (batch number: 210941, 208,051). Superoxide dismutase (SOD), Malondialdehyde (MDA), Glutathione peroxidase (GSH-PX) and Total antioxidant capacity (T-AOC) kit were purchased from Nanjing Jiancheng Bioengineering Institute (catalogue number: A001-1-2, A003-1-2, A005-1-2, A015-2-1). β-action, Protein kinase B (AKT) and

Goat anti-Rabbit IgG Antibody were purchased from Cell Signaling Technology (catalogue number: 4967S, 9272S, 7074S).

Instrument

Fully automatic biochemical analyzer (Beckman Coulter Laboratory Systems Co., Ltd., model: AU480, USA); MP150 multi-channel physiological signal acquisition and processing system (BIOPAC, USA); BioTek Epoch2 (American BioTek Instrument Co., Ltd., USA); 3K15 low temperature centrifuge (SIGMA, Germany); ZS-RCT-200 hot sting instrument (Beijing Zhongshi Dichuang Technology Development Co., Ltd., China); BX61 Optical microscopy Mirror (OlymPus, Japan); TP1020 automatic tissue dewatering processor (Leica, Germany); ST5010 Automatic tissue section dyeing machine (Leica, Germany); ODYSSEY Fc two-color fluorescence and luminescence imaging Systems (LI-COR Biosciences, USA); JY300HE electrophoresis apparatus (Beijing Junyi Dongfang electrophoresis Equipment Co., Ltd., China). AB Triple TOF6600 Mass Spectrometer (SCIEX, USA); 1290 Infinity LC Ultra-High Performance Liquid Chromatography (UHPLC) System (Agilent, USA); ACQUITY UPLC BEH Amide1 column (Waters Corporation, USA).

Study on Network Pharmacology of YXRJP

Active Ingredients and Target of YXRJP

Using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, https://old.tcmsp-e.com/tcmsp.php) database,[32] according to the conditions of "drug oral bioavailability (OB) ≥30% and drug similarity (DL) ≥0.18", all the Chinese medicinal materials of YXRJP were searched to screen out the eligible active ingredients and target data.

The Potential Targets of YXRJP in the Treatment of DPN

Using "diabetic peripheral neuropathy" as the search keyword, relevant targets of DPN were retrieved from GeneCards (www.genecards.org),[33] Online Mendelian Inheritance in Man (OMIM, https://omim.org/),[34] Disgenet (https://www.disgenet.com),[35] and DrugBank (https://go.drugbank.com/) databases.[36] The intersections between the targets of the active ingredients of YXRJP and those of DPN were collected to explore the potential targets of YXRJP for the treatment of DPN.

To Screen the Core Targets of YXRJP in the Treatment of DPN

The drug-disease intersection targets were imported into the STRING (https://cn.string-db.org/) database for protein-protein interactions (PPI) analysis. In this analysis, the research species was limited to "Homo sapiens". With the highest confidence level being higher than 0.9 and other parameters remaining at their default settings, the PPI network of YXRJP in the treatment of DPN was obtained.[37] Cytoscape3.8.2 software was used to construct the compound-target network of YXRJP for the treatment DPN, and according to degree values, a topological analysis was performed to analyze the key target proteins.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis of Intersection Targets

Using the DAVID (https://david.ncifcrf.gov/) database, "Homo sapiens" was selected as the species, GO enrichment analysis and KEGG pathway enrichment analysis were performed on the common target proteins of YXRJP and DPN.[38,39] The enrichment analysis results of GO function and KEGG signaling pathway were visualized by Omicshare platform (http://omicshare.com/).

Animals

According to the existing literature[40] and reference books,[41,42] it is essential that the number of experimental animals in each group complies with statistical requisites. In pharmacodynamic metrology statistics experiments, a minimum of 8 rats per group is typically required. In omics experiments, minimizing differences among individuals is of paramount importance for the accurate interpretation of experimental results and the derivation of precise conclusions.

Consequently, metabolomics analysis demands a high number of biological replicates. Simultaneously, during the experimental process, we factored in the potential occurrence of animal deaths or data loss. As a result, a final determination of 10 rats per group was made.

The results of our previous preliminary experiments showed that after injecting 2% STZ twice via the tail vein at a dose of 25mg/kg, the overall diabetes model establishment rate of rats was 75%. Therefore, considering the issue of whether the model would be successfully established or not, we used a total of 80 rats.

Eighty healthy male Sprague Dawley (SD) rats of specific pathogen free (SPF) status, weighing between 180 and 200 grams, were procured from Beijing Vital River Laboratory Animal Technology Co., Ltd, and the license number for experimental animal production is SCXK (Beijing) 2016–0011. Permit number for experimental institution is SYXK (Beijing) 2018–0008. Experimental animals were kept in an SPF animal room with fluorescent lighting, 12h light and dark cycle, free drinking and eating, temperature 20°C-26°C, air supply 6–10 times /h, and humidity 40–70%RH. This study was approved by Experimental Animal Ethics Committee of Beijing Tongrentang Research Institute (The ethical approval number: YJY-2021-031001).

Molding, Grouping and Drug Administration

After 4 days of adaptive feeding, 10 of 80 healthy male SD rats were randomly selected as the control group and fed with standard diet. The other 70 rats were fed with high-fat diet (45kcal% fat calories). Seven weeks later, all rats were fasted but allowed free access to water for 16 hours. Subsequently the rats fed with high-fat diet were intravenously injected 2% STZ at the dosage of 25mg/kg. The 2% STZ solution was prepared with citrate/sodium citrate buffer with pH4.5. The control group were intravenously injected with citrate/citrate sodium buffer at the same dose. Seven days later, blood samples were taken from the tail tip to determine the blood GLU level (blood GLU ≥16.7mmol/L). The rats that did not reach the target were injected with the same dosage of STZ again.[43–45] Seven days later, the blood GLU level was measured again. According to the blood GLU level, the diabetic model rats were randomly divided into the model group (diabetes control group), Mecobalamin group (30mg/kg), the high dose of YXRJP group (7.2g/kg, YXRJP-H), the middle dose of YXRJP group (3.6g/kg, YXRJP-M) and the low dose of YXRJP group (1.8g/kg, YXRJP-L). There were 10 rats in each group. Rats in each group were continuously given gavage for 12 weeks. Gavage volume: 10 mL/kg. The rats in the control group and the model group were given the same volume of animal drinking water by gavage for 12 weeks. The general condition of rats was observed every day. The body weight of rats in each group was weighed every two weeks, and the corresponding dosage was adjusted. Modeling and in vivo experimental design of rats with DPN are shown in Figure 1.

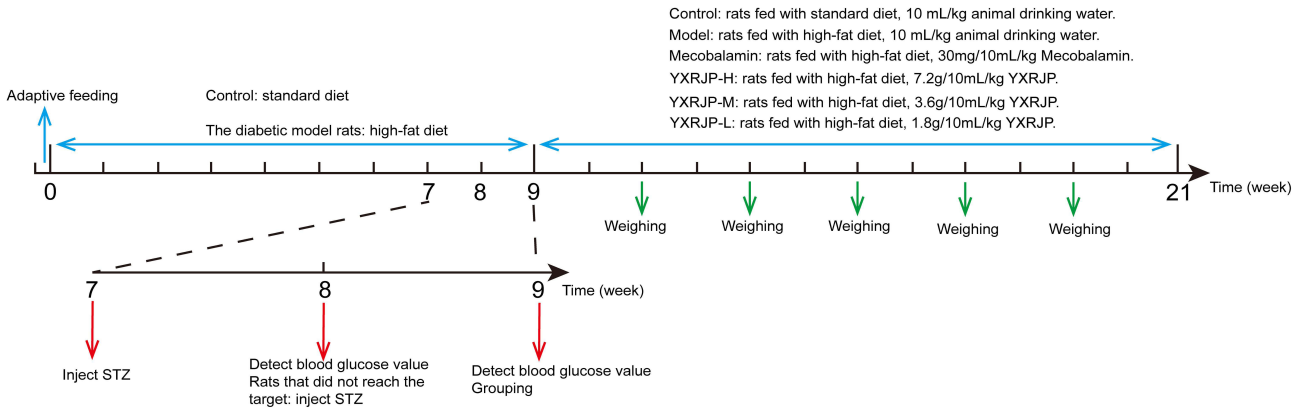


Figure 1 Modeling and in vivo experimental design of rats with DPN.

https://doi.org/10.2147/DDDT.S473146

Dynamic Measurement of Thermal Withdrawal Latency (TWL)

Before administration and 4, 8, 12 weeks after administration, the TWL of the rats in each group was measured with a thermal sting instrument. The left and right feet were tested three times in turn, and the average reaction time of each test was calculated as TWL.

Sciatic Motor Nerve Conduction Velocity (MNCV) Measurement

After the last administration, the rats in each group were anesthetized, and the sciatic nerve in the piriformis muscle was exposed through a 3 cm longitudinal incision along the posterior femoral part of the right lower limb under sterile conditions. The stimulating double-needle electrode was placed at the right ischial notch, and the recording electrode was placed between the second toes of the right foot. The electrophysiological signals were recorded after a single pulse square wave stimulation with 0.1m/s width, 1.5 times the threshold of stimulation intensity, and an interval of 5s or more. The latency difference (Δt) of action potential between the two channels was measured. MNCV (m/s) = the distance between stimulating electrode and recording electrode/latency. During the experiment, the room temperature was strictly controlled at about 25°C, and the body temperature of the animals was maintained at about 37°C.[46–50]

Determination of Serum Biochemical Indicators

Blood samples were collected from the abdominal aorta after MNCV was measured, and serum samples were collected after centrifugation at 3500rpm for 10min. The content of GLU was assessed with the GLU oxidase method. The content of TG was determined by Glycerol phosphate oxidase-phenol aminoantipyrine (Gpo-pap) method. The levels of GLU and TG in serum were determined by automatic biochemical analyzer.

Pathological and Ultrastructural Observation of Sciatic Nerve Tissue

One part of the sciatic nerve tissue was fixed in 2.5% glutaraldehyde for pathological ultrastructure examination by transmission electron microscopy, and the other part was fixed in 4% paraformaldehyde for immunohistochemical examination.

Determination of Oxidative Stress Factors

After the animals were anesthetized, blood was collected from the abdominal aorta. The activity of GSH-PX was evaluated by substrate consumption method. The level of T-AOC was evaluated by 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) method. The activity of SOD was evaluated by xanthine oxidase method. The content of MDA was determined by condensation method with thiobarbituric acid. The activity/content of GSH-PX, T-AOC, SOD and MDA in the serum of the rats was determined according to the operation instructions of the kit.

Measurement of IGF-1 Expression in Sciatic Nerve Tissue

The sciatic nerve tissue of rats was fixed and routinely embedded in paraffin, sliced, dewaxed, antigen repaired and blocked with specific reagents and then subjected to staining treatment. The images were observed under microscope and collected. Use Image J to analyze and process images and calculate the integrated optical density (IOD) value of the positive area.[51]

Determination of Relative AKT and IGF-1 Protein Expression in Sciatic Nerve Tissue

Western Blot was used to detect the expression of AKT and IGF-1 protein in the sciatic nerve tissue of rats with DPN. 40μg of protein samples were taken for electrophoresis at 200V for 30min, and the Nitrocellulose (NC) membrane was soaked in electrotransfer at 300mA for 60min. The NC membrane was placed in the blocking solution for 2h at room temperature, and washed 3 to 5 times with TBST for 5 min each time. The NC membrane was shaken in the primary antibody for overnight at 4°C, and washed three times with TBST for 5min each time. Membranes were incubated in secondary antibodies in the dark for 1h, and washed 3 to 5 times with TBST for 5min each time. NC membranes were scanned using the Odyssey Imaging System, and the scans were saved and analyzed.

Plasma Metabolomics Studies

Plasma Sample Processing

Blood samples (1.5mL) were collected from the abdominal aorta of rats and placed in a 10% heparin anticoagulant centrifuge tube at 3000 rpm for 10 min. After centrifugation, the supernatant was separated into centrifuge tubes and stored in an ultra-low temperature refrigerator at −80°C. After the sample was thawed slowly at 4°C, an appropriate amount of sample was added to the precooled methanol/acetonitrile/water solution (2: 2: 1, v/v), vortexed and mixed, sonicated at low temperature for 30min, placed at −20°C for 10min, centrifuged at 14000×g at 4°C for 20min, took the supernatant and dried under vacuum, and added 100μL aqueous acetonitrile solution to mass spectrometry (acetonitrile: water = 1: 1, v/v), vortexed, centrifuged at 14000×g for 15min at 4°C, and the supernatant was injected for analysis.

Chromatographic and Mass Spectrometry Conditions

The samples were separated on Agilent 1290 Infinity LC UHPLC Hydrophilic Interaction Liquid Chromatography (HILIC) column. The column temperature was 25°C. The flow rate was 0.5mL/min; The injection volume was 2μL. Mobile phase composition A: water +25mM ammonium acetate +25mM ammonium hydroxide, B: acetonitrile; The gradient elution procedure was as follows: 0–0.5min, 95%B; 0.5–7min, B changed linearly from 95% to 65%; 7–8min, B changed linearly from 65% to 40%, 8–9min, B maintained at 40%; 9–9.1min, B changed linearly from 40% to 95%; 9.1–12min, B was maintained at 95%; The samples were placed in an autosampler at 4°C throughout the analysis. In order to avoid the influence caused by the fluctuation of the instrument detection signal, the samples were consecutively analyzed in random order. QC samples (QC samples were made by mixing equal amounts of samples to be tested) were inserted into the sample cohort to monitor and evaluate the stability of the system and the reliability of the experimental data.

The primary and secondary spectra of the samples were collected by AB Triple TOF 6600 Mass Spectrometer. Samples were separated on an Agilent 1290 Infinity LC UHPLC system and analyzed by AB Triple TOF 6600 Mass Spectrometer using electrospray ionization (ESI) positive ion and negative ion modes, respectively. ESI source setting parameters are as follows: atomizing gas auxiliary heating gas 1 (Gas1): 60, auxiliary heating gas 2 (Gas2): 60, curtain gas (CUR): 30psi, ion source temperature: 600°C, ion-source voltage (ISVF) is +5500 V in positive mode and −5500 V in negative mode. The detection range of the primary mass-to-charge ratio: 60–1000Da, the secondary daughter ion mass-to-charge ratio: 25–1000Da, the primary mass spectrum scanning accumulation time: 0.20s/spectra, the secondary mass spectrum scanning accumulation time: 0.05s/spectra. The secondary mass spectra were obtained by data dependent acquisition (DDA) mode, and the peak intensity value screening mode was used. The declustering potential (DP) was +60V in positive mode and −60V in negative mode, and the collision energy was 35 ± 15eV. The DDA Settings were as follows: the dynamic exclusion isotope ion range was 4Da, and 10 fragments were collected for each scan.

Analysis of Potential Biomarkers

Metabolic profile analysis was performed using SIMCA-P software package and R language. Principal Component Analysis (PCA) with unsupervised pattern recognition was performed on the data matrix. PCA and Orthogonal Partial Least Squares Discrimination Analysis (OPLS-DA) with supervised pattern recognition were used to obtain an overall representation of the distribution of data samples and differences between groups. To achieve overall discrimination of the metabolic profiles of two or more groups of samples. Then OPLS-DA was used to determine the metabolic differences between groups. According to the variable important in projection (VIP) and the P value from a two tailed Student's t-test, differential metabolites with VIP > 1 and P value < 0.05 were considered as potential biomarkers.

Data Processing and Analysis

Data were processed by SPSS 27.0 software system and R packages (version: 4.4.2).[52,53] We performed the Shapiro–Wilk test and Q-Q plots for assessing normality. All the data were expressed as mean ± SD. For the comparison between two groups, Student's t-tests were used considering the normally distributed data. Among multiple groups, comparisons

were performed using one-way analysis of variance (ANOVA) on the normally distributed data, the least significant difference (LSD) tests were used for post hoc analysis. The Kruskal–Wallis *H*-test was used when the data had a non-normal distribution, then the Dunn's pairwise comparison test was carried out as a post hoc test. Values of $P < 0.05$ were considered statistically significant.

Results

Network Pharmacology Analysis of YXRJP in the Treatment of DPN

Prediction of Disease Targets of YXRJP in the Treatment of DPN

The 459 targets corresponding to active ingredients in YXRJP screened from the TCMSP database were intersected with DPN disease targets, and a total of 301 intersection targets were obtained. The results are shown in Figure 2A.

Construction and Analysis of Drug-Ingredient-Target Network Model of YXRJP

Cytoscape3.8.2 was used to construct the "drug-ingredient-target" network of YXRJP in the treatment of DPN, with a total of 301 potential targets, and the results are shown in Figure 2B.

Construction of PPI Network and Analysis of Core Targets

The 301 targets obtained from the drug-disease intersection were imported into the STRING database for PPI analysis. In the PPI network diagram, the more the number of connections between proteins, the higher the degree of mutuality, indicating that the pharmacological effect of this protein in the treatment of diseases is more obvious, the results are shown in Figure 2C. The protein interaction relationship data were introduced into Cytoscape3.8.2 software, and the target genes were screened by adjusting the parameters such as Betweenness, Closeness, Degree and so on. The target genes larger than the median value were retained to obtain subnetwork 1. Subnetwork 1 was screened again to obtain subnetwork 2. Subnetwork 3 was obtained by screening, and a total of 13 core targets were found, and the network relationship is shown in Figure 2D.

GO Functional Enrichment Analysis

DAVID database was used to perform GO enrichment analysis of the common target proteins (301 potential target proteins) of YXRJP and DPN. Functional Annotation Clustering was used to perform biological process GO enrichment analysis of biological process (BP), molecular function (MF) and cellular component (CC) which might exist in the effect of YXRJP ($p < 0.05$). The enriched signaling pathways of the common targets of YXRJP and DPN could be obtained. The analysis of Classification Stringency selection Medium and GO functional enrichment obtained 872 GO entries ($p < 0.05$), of which 664 items were BP entries, 130 items were MF entries and 78 items were CC entries. The top 20 items were taken as the bar chart, and the results are shown in Figure 3A. The results suggest that YXRJP may regulate the response of cells to hypoxia, inflammation and lipopolysaccharide by affecting the activity of protein serine/threonine kinase and steroid hormone receptor in DPN.

KEGG Pathway Enrichment Analysis

Using the DAVID database, "Homo sapiens" were selected to perform KEGG pathway enrichment of the common target proteins of YXRJP and DPN, and the corrected *P* value was set to be less than 0.05. A total of 140 signaling pathways were identified. List the top 20 according to the adjusted *P* value from lowest to highest. The pathways related to DPN disease include Insulin resistance, Toll-like receptor (TLR) signaling pathway, Tumor necrosis factor (TNF) signaling pathway, and Non-alcoholic fatty liver disease signaling pathway. The results are shown in Figure 3B.

Pharmacodynamic Evaluation of YXRJP in the Treatment of DPN

Effects of YXRJP on Body Weight in DPN Rats

After the diabetic rat model was established by feeding with high-fat diet combined with STZ injection, the rats showed obvious polydipsia, polyuria and weight loss. After 4 weeks of administration, compared with the model group, the body weight of the rats in YXRJP-M group increased significantly ($P < 0.05$). After 8 weeks of administration, the body

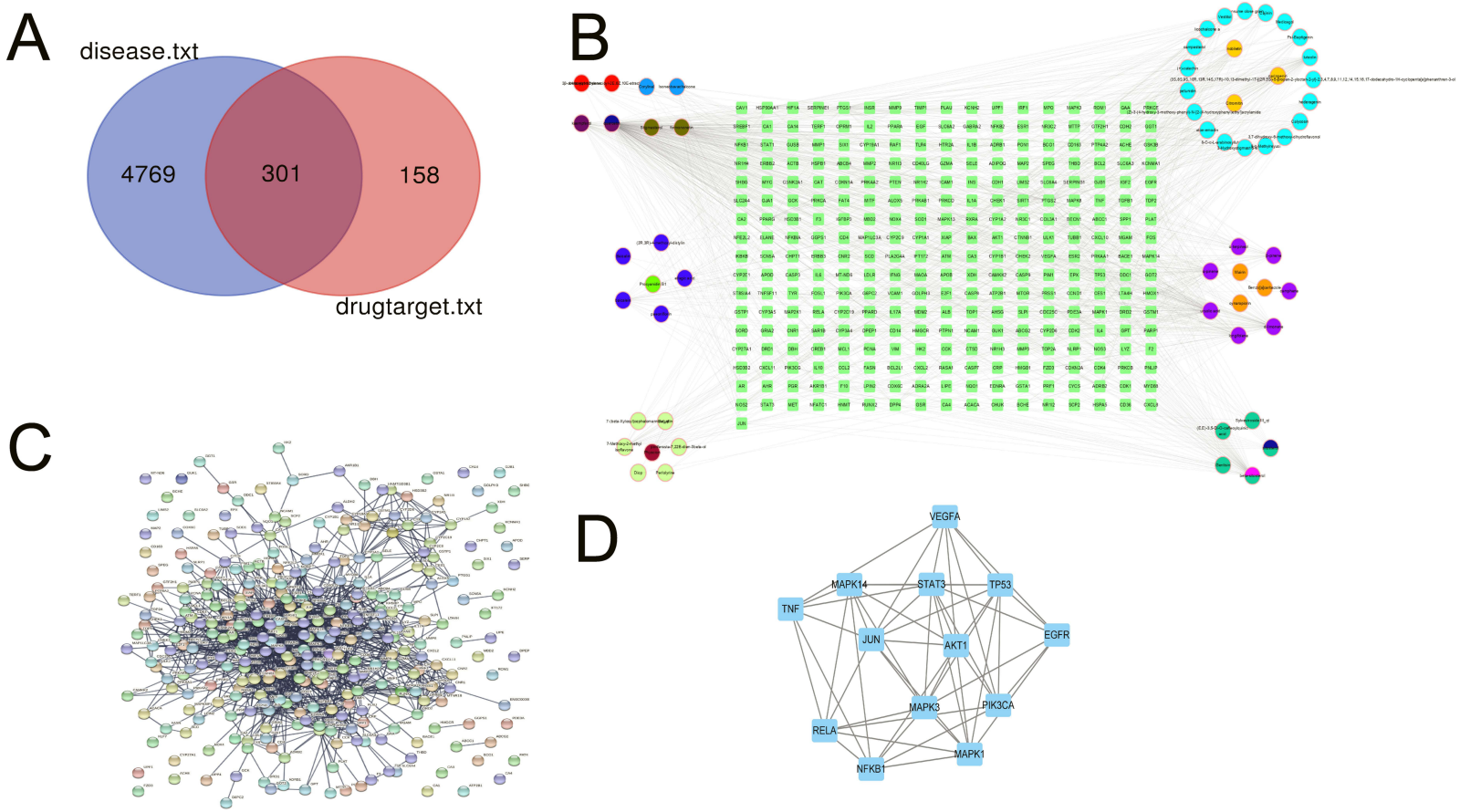


Figure 2 Related targets information of YXRJP in the treatment of DPN. (**A**) Veen map of targets at the intersection of YXRJP and DNP. (**B**) Diagram of the drug-ingredient-target network. (green nodes: proteins of target, light blue nodes: the active ingredient of *Spatholobus suberectus* Dunn, yellow nodes: the active ingredient of *Citrus reticulata* Blanco, purple nodes: the active ingredient of *Pinus massoniana* Lamb., Orange nodes: the active ingredient of *Aucklandia lappa* Decne., blue-green nodes: the active ingredient of *Dipsacus asper* Wall. ex C.B. Clarke, deep blue nodes: the active ingredient of *Taxillus sutchuenensis* (Lecomte) Danser, pink nodes: the active ingredient of *Angelica sinensis* (Oliv.) Diels, red nodes: the active ingredient of *Atractylodes macrocephala* Koidz., blue nodes: the active ingredient of *Psoralea corylifolia* L., deep purple nodes: the active ingredient of *Phryma leptostachya* L., atrovirens nodes: the active ingredient of *Lycopodium japonicum* Thunb., bluish violet nodes: the active ingredient of *Paeonia anomala* L., peak green nodes: the active ingredient of *Vigna umbellata* (Thunb.) Ohwi & H.Ohashi, light green nodes: the active ingredient of *Codonopsis pilosula* (Franch.) Nannf., deep red nodes: the active ingredient of *Pleuropterus multiflorus* (Thunb.) Turcz. ex Nakai.) (**C**) PPI network. (**D**) The core targets of YXRJP in the treatment of DPN.

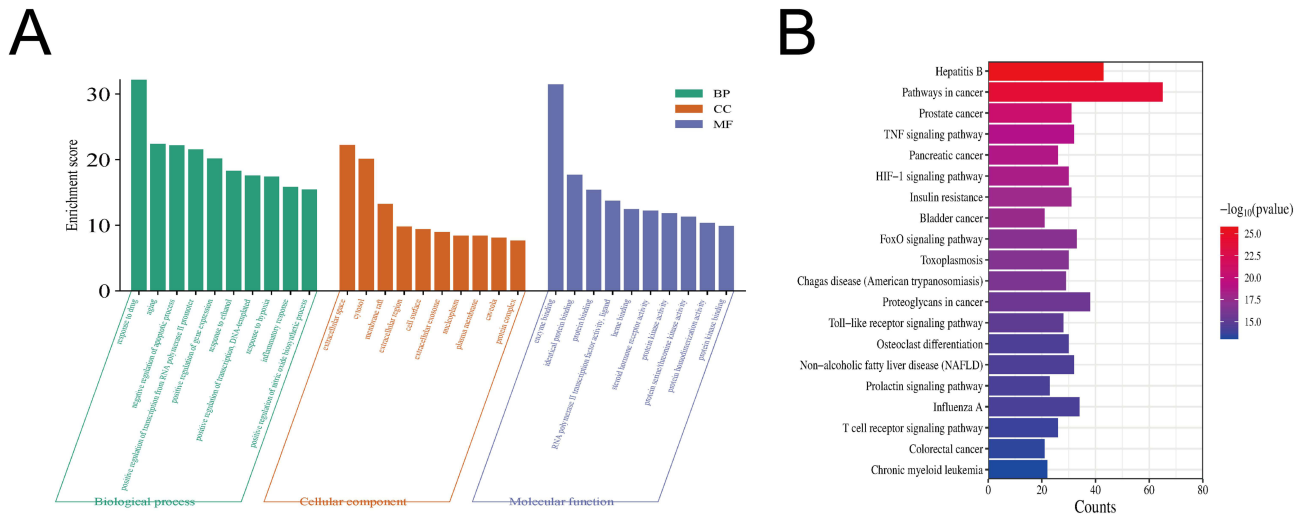


Figure 3 Pathway of action of YXRJP in the treatment of DPN. (**A**) GO enrichment analysis of DPN treated with YXRJP. (**B**) KEGG enrichment analysis of DPN treated with YXRJP.

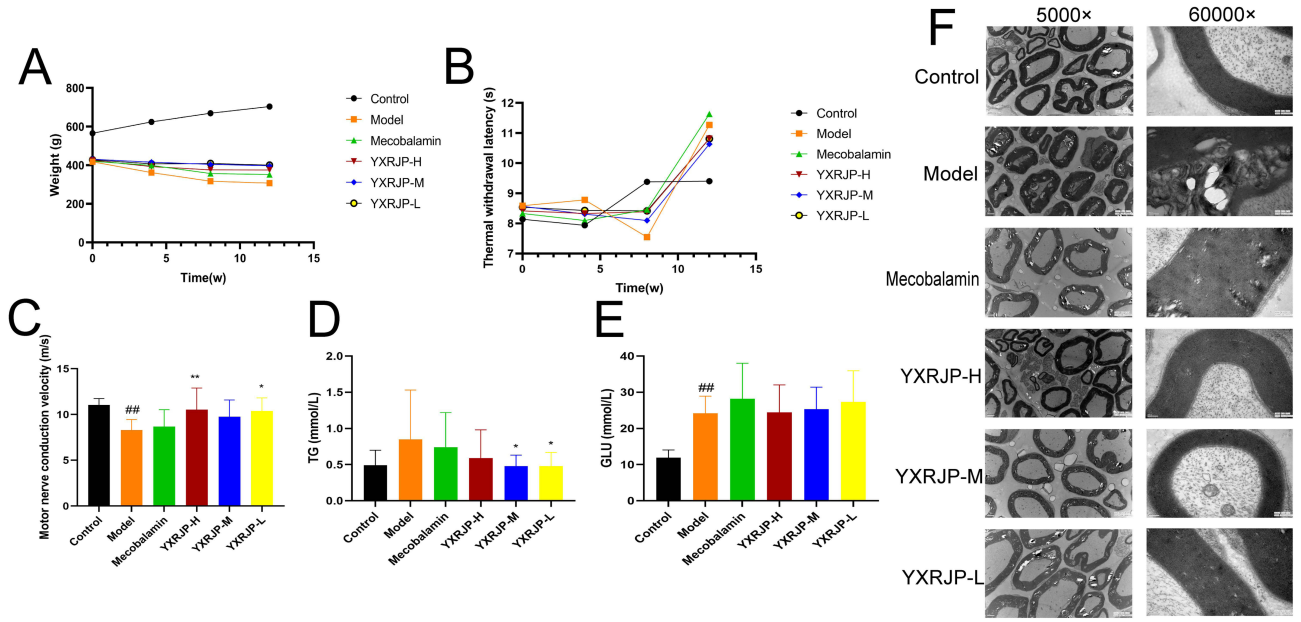


Figure 4 Pharmaceutical Effect of YXRJP in DPN rats. (**A**) Effect of YXRJP on body weight in DPN rats, *n*=10. (**B**) Effect of YXRJP on TWL in DPN rats, *n*=10. (**C**) Effect of YXRJP on sciatic MNCV in DPN rats, *n*=8. (**D**) Effect of YXRJP on TG in DPN rats, *n*=10. (**E**) Effect of YXRJP on GLU in DPN rats, *n*=10. (**F**) Effect of YXRJP on the ultrastructure of sciatic nerve in DPN rats. The data were presented as mean ± SD. $^{\#\#}P < 0.01$, vs Control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs Model group.

weight of the rats in the YXRJP-H, YXRJP-M, and YXRJP-L groups increased significantly ($P < 0.05$ or $P < 0.01$), and the results are shown in Figure 4A.

Effects of YXRJP on TWL in DPN Rats

After 8 weeks of administration, compared with the control group, the TWL of the model group was significantly decreased ($P < 0.01$), and the TWL of the Mecobalamin group and each dose of YXRJP group was increased, but there was no significant difference. After 12 weeks of administration, the TWL of the model group was significantly higher than that of the control group ($P < 0.01$). Compared with the model group, the TWL of the rats in each dose group of YXRJP was decreased to some degree, but there was no statistical significance. The results are shown in Figure 4B.

Effect of YXRJP on Sciatic MNCV in DPN Rats

Compared with the control group, the sciatic MNCV in the model group was significantly decreased ($P < 0.01$). After administration, compared with the model group, the sciatic MNCV of the rats in the YXRJP-H and YXRJP-L groups was significantly increased ($P < 0.05$ or $P < 0.01$), and the results are shown in Figure 4C.

Effects of YXRJP on Serum Biochemical Levels in DPN Rats

Compared with the control group, the serum level of GLU in the model group was significantly increased ($P < 0.01$). After drug intervention, compared with the model group, there was no significant difference in GLU level in each group of YXRJP. The TG level of rats in the YXRJP-M and YXRJP-L groups decreased significantly ($P < 0.05$). The results are shown in Figure 4D and E.

Effect of YXRJP on the Ultrastructure of Sciatic Nerve Tissue in DPN Rats

As shown in Figure 4F, the axons of sciatic nerve fiber in the control group were surrounded by myelin sheath. The myelin sheath was in concentric circles, had clear lamellar structure and uniform density, and the microtubules and microfilaments were neatly arranged. In the model group, the myelin lamina structure of a large number of myelinated nerve fibers in the sciatic nerve was seriously damaged, the lamina was blurred, and a large number of vacuole-like defects appeared. A large number of separated myelin fibers protruded into the axons to form tumorlike structures, axons were deformed and shrunk, microtubules and microfilaments were arranged disorderly, and cracks between nerve fibers increased. The vacuolated loss of myelin sheath in the sciatic nerve tissue of the rats in the YXRJP-H and YXRJP-M groups was significantly reduced, the lamellar structure was clear, the unmyelinated nerve bundles were significantly increased, and the microfilaments and microtubules were clearly arranged, suggesting that YXRJP could significantly alleviate the ultrastructure damages of the sciatic nerve tissue in this model.

Effects of YXRJP on Oxidative Stress Related Factors in DPN Rats

Compared with the control group, the levels of SOD and T-AOC in serum of the model group were decreased, but there was no significant difference. The level of GSH-PX was decreased significantly ($P < 0.05$), and the level of MDA was increased, but there was no significant difference. Compared with the model group, the activity of SOD in the serum of rats in each dose group of YXRJP was significantly increased ($P < 0.01$). The GSH-PX level of rats in the YXRJP-M and YXRJP-L groups was significantly increased ($P < 0.05$). The MDA level was significantly decreased in the YXRJP-H group ($P < 0.05$), and the results are shown in Figure 5A–D.

Effect of YXRJP on the Expression of IGF-1 in Sciatic Nerve Tissue of DPN Rats

IGF-1 was mainly distributed in the cytoplasm and blood vessels. Immunohistochemical results showed that the expression of IGF-1 protein in the sciatic nerve tissue of the control group was strongly positive, and the expression of IGF-1 protein in the sciatic nerve tissue of the model group was weakly positive. Compared with the control group, the expression of IGF-1 protein in the sciatic nerve tissue of the model group was significantly decreased ($P < 0.01$). Compared with the model group, the expression of IGF-1 protein in the sciatic nerve tissue of rats in each dose group of YXRJP was significantly increased ($P < 0.05$). The results are shown in Figure 5E and F.

Effects of YXRJP on the Expression of AKT and IGF-1 Protein in Sciatic Nerve Tissue of DPN Rats

The expression of IGF-1 protein in the sciatic nerve tissue of the model group was significantly decreased ($P < 0.01$), and the expression of AKT protein was increased ($P > 0.05$). After administration, different doses of YXRJP could promote the expression of neuroprotective proteins to varying degrees, and inhibit the expression of inflammation-related proteins to some extent. Compared with the model group, the expression of IGF-1 protein in the sciatic nerve tissue of rats in each dose group of YXRJP was significantly increased ($P < 0.05$), and the expression of AKT protein was significantly decreased ($P < 0.05$ or $P < 0.01$). The results are presented in Figure 5G–I.

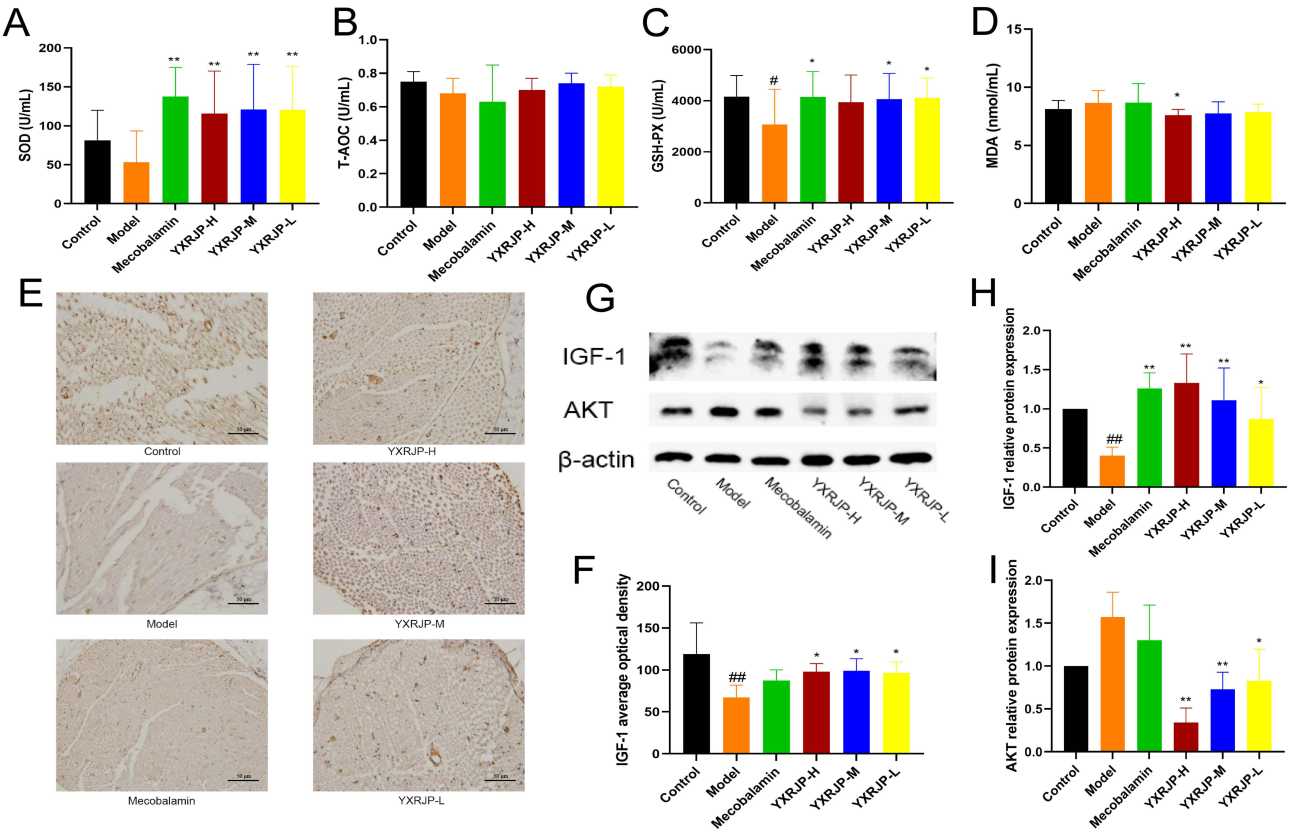


Figure 5 Mode of action of YXRJP in DPN rats. (**A-D**) Effect of YXRJP on oxidative stress in DPN rats, *n*=10. (**E** and **F**) Effect of YXRJP on IGF-1 protein expression in sciatic nerve of DPN rats, *n*=10. (**G-I**) Effect of YXRJP on the relative expression of IGF-1 protein and AKT protein in sciatic nerve of DPN rats, *n*=6. The data were presented as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs Control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs Model group.

Study on Plasma Metabolomics of YXRJP in DPN Rats

Experimental Quality Control

In the positive and negative ion detection mode, the total ion current map (TIC) of all QC samples showed that the response intensity and retention time of each chromatographic peak basically overlapped, indicating that the instrument analysis system in this test had good stability, the test data were stable and reliable, and the differences in metabolic spectra obtained in the test could reflect the biological differences among the samples. The specific results are shown in Figure 6A and B.

Comparison of Global Metabolic Profiles in Rat Plasma

PCA was used to compare the metabolic profiles of all samples. The results showed that the plasma metabolic profile of the model group was significantly different from the control group. There may be an overlap between the YXRJP group and the model group. To identify the different metabolites in each group and maximize the separation between groups, the supervised pattern recognition method OPLS-DA was applied. In the positive and negative ion modes, the groups can be well separated and clustered within the groups. The results are shown in Figure 7A–L.

To avoid overfitting of the OPLS-DA model during the modeling process, we conducted permutation tests for verification. R2 represents the fitting of the model to known data. The closer R2 is to 1, the better the model fits the data and the more information it can explain. Q2 represents the predictive ability of the model for new data. The closer Q2 is to 1, the stronger the predictive ability of the model. As shown in Figure 7C, F, I and L the abscissa represents the degree of permutation retention, and the ordinate represents the values of R2 and Q2. As the degree of permutation retention gradually decreases, both R2 and Q2 of the random model gradually decline. The results indicated that there was no overfitting phenomenon in the original model, and the model had good robustness.

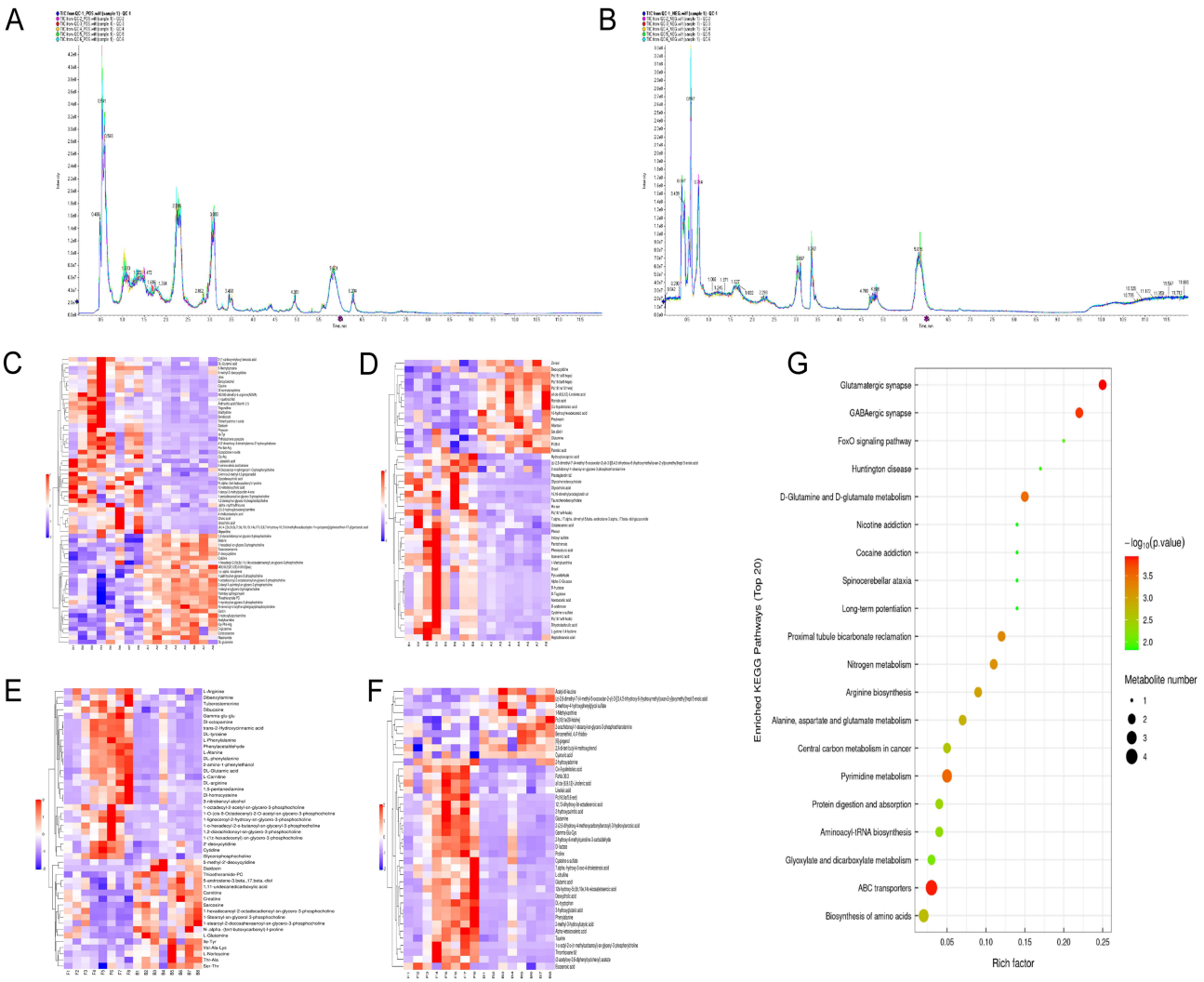


Figure 6 TIC of QC samples (**A** and **B**) and differential metabolites and metabolic pathways analysis in Plasma between blank group, model group and YXRJP group (**C-G**). (**A**) TIC of QC samples in positive ion mode. (**B**) TIC of QC samples in negative ion mode. (**C** and **D**) cluster analyses between the Control and Model group, *n*=8. (**C**) ESI, (**D**) ESI. (**E and F**) cluster analyses between the model and YXRJP group, *n*=8. (**E**) ESI, (**F**) ESI. (**G**) Bubble plot analysis of KEGG enrichment of potential biomarkers.

https://doi.org/10.2147/DDDT.S473146

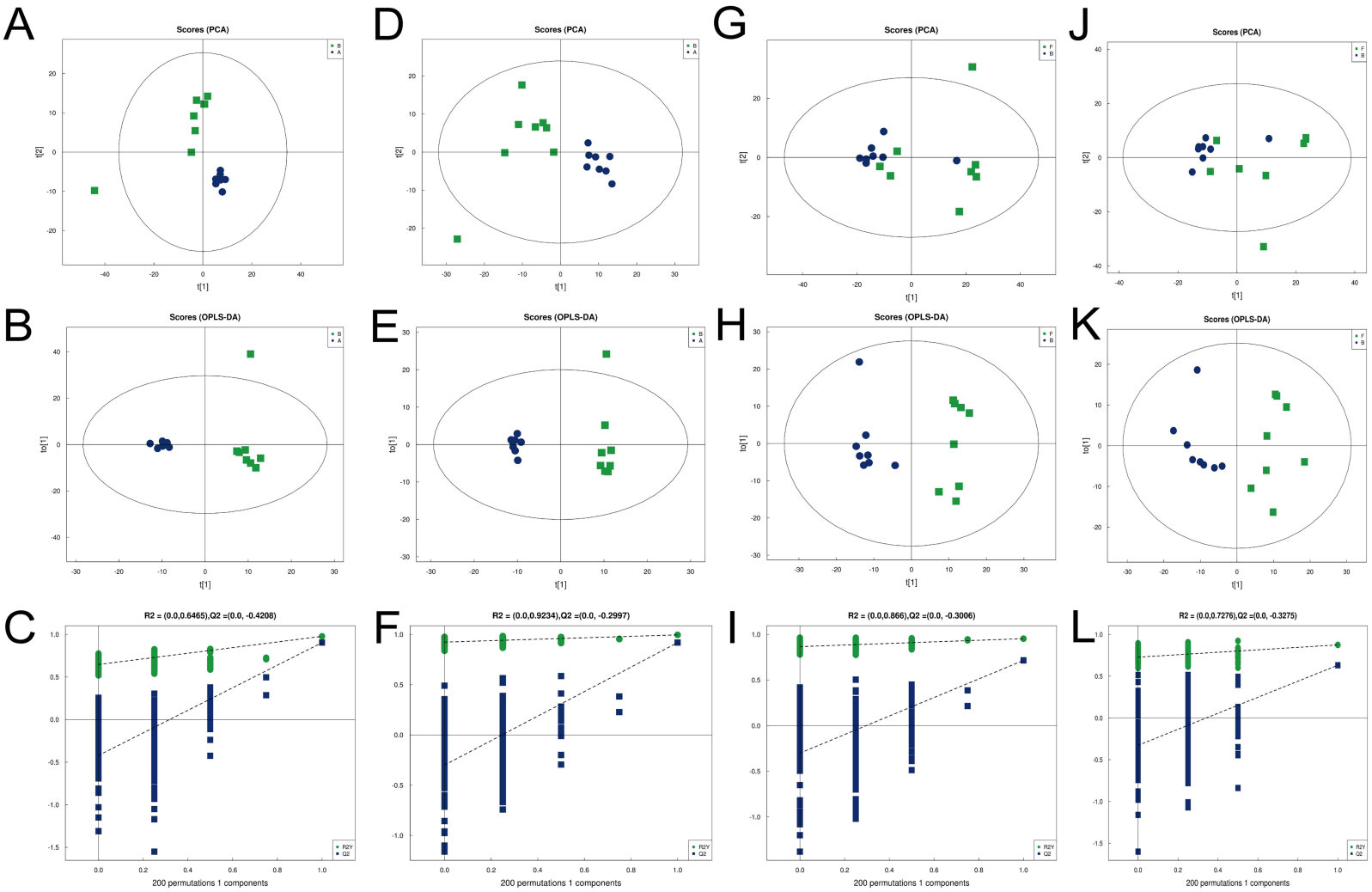


Figure 7 PCA and OPLS-DA and permutation score plots. (**A–C**) plasma sample of the control and model group, ESI, *n*=8. (**D–F**) plasma sample of the control and model group, ESI, *n*=8. (**G–I**) plasma sample of the model and YXRJP group, ESI, *n*=8. (**J–L**) plasma sample of the model and YXRJP group, ESI, *n*=8.

Table 1 Intersection of Potential Biomarkers of the Control, Model and YXRJP Group

number	Intersection of significantly different metabolites	B vs A	F vs B
1	2'-deoxycytidine	↓	↑
2	Cytidine	↓	↑
3	Tuberostemonine	↓	↑
4	Daidzein	↑	↓
5	N-.alpha.- (tert-butoxycarbonyl) -l-proline	↑	↓
6	Ile-Tyr	↑	↓
7	5-methyl-2'-deoxycytidine	↑	↓
8	1,2-diarachidonoyl-sn-glycero-3-phosphocholine	↓	↑
9	Cis-9-palmitoleic acid	↓	↑
10	All cis- (6,9,12) -Linolenic acid	↓	↑
11	1-Methylxanthine	↑	↓
12	Glutamine	↓	↑
13	(z) −2,6-dimethyl-7- (4-methyl-5-oxooxolan-2-yl) −3-[[3,4,5-trihydroxy-6- (hydroxymethyl) oxan-2-yl]oxymethyl] hept-5-enoic acid	↑	↓
14	2-arachidonoyl-1-stearoyl-sn-glycero-3-phosphoethanolamine	↑	↓

Note: B vs A indicates comparison between the model and control group. F vs B indicates comparison between the YXRJP and model group.

Analysis of Potential Biomarkers and Metabolic Pathways

110 differential metabolites between the control group and model group and 86 differential metabolites between the model group and YXRJP group were screened out under the conditions of OPLS-DA with VIP > 1 and P value < 0.05. The overall levels of 14 metabolites, including 5-methyl-2 '-deoxycytidine, cis-9-palmitoleic acid, all-cis - (6,9,12) - linolenic acid and glutamine, were adjusted more to the control group after the intervention of YXRJP. The results are shown in Figure 6C–F.

Biological processes are usually composed of a group of genes rather than individual single genes.[54] Pathway enrichment analysis was used to observe a certain common trend shown by a group of related metabolites under experimental conditions. This can avoid, to some extent, the false positive or false negative results that may occur when only focusing on the changes of individual metabolites. Pathway enrichment analysis takes into account the functional correlations among metabolites, and the enrichment results are more biologically significant.[55] The potential metabolic pathways of the above differential metabolites were analyzed. The main involved metabolic pathways were those of arginine, alanine, aspartic acid and other amino acid metabolic pathways, as well as nucleotide metabolic pathways. The results are shown in Table 1 and Figure 6G.

Discussion

As one of the chronic complications of diabetes with the highest prevalence, DPN involves a wide range of pathophysiology, and encompasses almost all parts of the nervous system. In the early stage of the disease, the patient's self-conscious symptoms are not obvious, but nerve conduction disorders have already appeared, and then paresthesia occurs, including numbness, formication, and electric shock. The typical type is glove or sock distribution, and in severe cases, lower limb joint lesions and ulcers can occur.[56] In clinical practice, active GLU control can delay the development of DPN to a certain extent, but after the occurrence of DPN, even if GLU is effectively controlled, it is still difficult to recover. At present, clinical drug treatment can be divided into drugs to improve symptoms and drugs to treat the pathogenesis. Due to the safety problems caused by long-term use of drugs, the treatment plan is relatively conservative, in order to avoid side effects, limit the dose, and ultimately affect the treatment effect.[57]

According to TCM, DPN is caused by diabetes that has consumed Qi and Yin for a long time and gradually progresses to meridian obstruction. It belongs to "bi syndrome". DPN is caused by factors such as wind, cold, phlegm and dampness leading to blockage of meridians, resulting in malnutrition and blood stasis of meridians.[58] Studies have shown that TCM may affect the neuronal repair and regeneration of DPN. TCM prevent and treat DPN by increasing the levels of neurotrophic factors or their receptors and protecting neurons.[59] Blood stasis is also an important pathogenesis

of chronic vascular complications of diabetes.[60] Promoting blood circulation can effectively alleviate complications. TCM blood circulation activating drugs have obvious advantages in the prevention and treatment of diabetic microvascular complications.[61] According to the dynamic evolution of the course of the disease, DPN can be divided into three main stages: numbness, pain and muscle atrophy. For patients with early DPN, active intervention treatment can effectively alleviate symptoms and delay the further development of DPN.[62] Because TCM has the advantages of multi-component, multi-target and preventive treatment effect. In recent years, the treatment of DPN with TCM compound has achieved good effect.[63–65] YXRJP is composed of 16 herbs, including *Angelica sinensis* (Oliv). Diels, *Spatholobus suberectus* Dunn, *Pleuropterus multiflorus* (Thunb). Turcz. ex Nakai, *Paeonia anomala* L., which are used to enrich the blood, invigorate blood circulation, activating collaterals, relieve pain, and *Clematis chinensis* Osbeck, *Lycopodium japonicum* Thunb., *Phryma leptostachya* L. and *Pinus massoniana* Lamb., which are used to dispel wind and collaterals and relieve pain. It has the functions of nourishing blood and nourishing tendons, dispelling wind and activating collaterals. Based on the treatment principle of DPN, which is to invigorate Qi, nourish Yin, promote blood circulation, remove blood stasis, dispel dampness and dispel cold,[66] and considering the main symptoms such as pain, numbness and atrophy.[67]

This study carried out the secondary research and development of YXRJP for the exploratory study of the treatment of DNP. The results showed that YXRJP could treat DPN by improving rat symptoms, nerve damage, oxidative stress, inflammatory response, fatty acid metabolism, amino acid metabolism and nucleotide metabolism in rats. It reflects the advantages of multi-component, multi-target and multi-pathway of TCM compound. At the same time, in terms of medication safety, no related adverse reactions have been reported in the long-term clinical application of YXRJP. However, the mechanism of action and clinical efficacy still need to be further clarified.

Network pharmacology analysis predicted that YXRJP could treat DPN mainly through 13 core targets such as TP53, JUN, MAPK3, NFKB1 and AKT1. Studies have shown that Schwann cells, the glial cells of the peripheral nervous system, are able to pass attenuation transcription factor JUN promotes remyelination of peripheral nerves and improve demyelination in DPN.[68] MAPK1 retards DPN by inhibiting the activation of stress-activated protein kinases in the nervous system occurrence and development of lesions.[69] Luo et al found that the down-regulation of the MAPK signaling pathway may be the main pathogenesis of DPN.[70] Serine/threonine protein kinase 1 (AKT1) is one of AKT kinases. It can regulate cell survival, proliferation, metabolism and angiogenesis, and affect the proliferation, apoptosis of islet β cells and nerve cell regeneration.[71] It induces GLU transport and is responsible for regulating GLU uptake and regulating diabetes and its complications.[72] Studies have shown that AKT plays a key role in the pathogenesis of neuropathic pain and inflammatory pain. After administration of AKT inhibitors, the pain of animals with sciatic nerve injury was significantly reduced.[73] Wang et al also found that a decrease in AKT expression level can alleviate peripheral neuralgia in diabetic rats.[74–76] KEGG pathway enrichment analysis showed that YXRJP treats DPN mainly through TNF and TLR signaling pathways. TNF-α is produced by activated natural killer cells, macrophages, lymphocytes, mast cells and eosinophils. It causes toxic effects on neurons and glial cells and results in the damage of nerve cells and demyelination of nerve fibers. It also induces the secretion of IL-1β and IL-6 by stimulating vascular endothelial cells and monocytes, and then can aggravate the inflammation and damage in vascular endothelial cells.[77] The study found that in diabetic and DPN patients, TNF-α level is increased and the never myelin basic protein (MBP) expression is decreased. Recombinant human TNF-α receptor II-Fc fusion protein (rhTNFR:Fc) could improve the MNCV, fiber demyelination and axon structure disorder by inhibiting the expression of TNF-α.[78] TLRs are widely distributed on the surface of immune cells and epithelial cells. TLRs/NF-κB signaling pathway plays an extremely important role in immune regulation and inflammatory reaction.[79] The NF-κB activated by this pathway reaches the nucleus, binds to the corresponding promoter, and regulates the expression of various genes. Then it eventually activates the specific immune system and plays an important role in regulating immunity and cell proliferation.[80] Fan et al also confirmed that regulating the TLR/ NF-κB signaling pathway can significantly reduce the thermal and mechanical stimulation thresholds of diabetic mice and increase MNCV.[81] Based on the above analysis, this study predicts that YXRJP can treat the DPN through playing important roles in anti-inflammation, protecting nerve fibers, relieving diabetic neuropathic pain, inducing angiogenesis, improving microvascular circulation, recovering vascular function, and improving the ischemia and hypoxia state of neurocytes. These effects may be related to AKT1, MARK3, NFKB1 and other targets.

In the pathogenesis of DPN, small myelinated Aδ nerve fibers and unmyelinated C nerve fibers are the first to be affected, followed by large myelinated Aβ nerve fibers.[82] TWL is the reaction time to detect the injury caused by thermal stimulation in rats, which mainly expresses the function of unmyelinated and small myelinated nerve fibers. The results showed that the latency of thermal pain in the model rats decreased significantly after 8 weeks of modeling. With the extension of modeling time, the latency of thermal pain in the model rats increased gradually, and the sensation of thermal pain decreased. All doses of YXRJP can obviously adjust the abnormal latency of model rats. Transmission electron microscopy shows that YXRJP can improve the function of myelinated fibers. The results show that YXRJP has a certain improvement effect on abnormal heat pain sensitivity and sciatic nerve injury that occur during the development of DPN. Yang et al found that TCM can protect the sciatic nerve myelin sheath structure of DPN mice and improve the TWL and MNCV.[83] This discovery proves the potential of TCM in the treatment of DPN. MNCV measurement is an important method for the detection of DPN, and it is also the gold index for the diagnosis of peripheral neuropathy.[84] Compared with the model group, the sciatic MNCV of rats in each dose group of YXRJP was significantly increased, suggesting that YXRJP has obvious protective effect on the sciatic nerve function of rats with DPN.

Oxidative stress plays an important role in the process of DPN. A large amount of ROS directly causes lipid peroxidation on the membrane structure of nerve cells, damage the cell endoplasmic reticulum, lysosomes and mitochondria, destroy the integrity of cell structure, change cell permeability and finally induce the loss of cellular function. Previous studies have found that abnormal expression of SOD and GSH-PX is an important part of the pathophysiology of diabetic microvascular complications.[85] IGF-1 is a kind of active protein polypeptide, which is the autocrine and paracrine product of a variety of cells in the body. It has multiple effects such as promoting axonal growth, reducing neuronal apoptosis, and promoting myelin regeneration. Song et al found that TCM compound prescriptions can promote the expression of IGF-1 protein in the sciatic nerve to improve the symptoms of DPN rats.[86] In this study, it was found that YXRJP could increase the activity of SOD and the level of GSH-PX in the serum of model rats, reduce the level of MDA, inhibit the oxidative stress response of DPN rats, and increase the expression of IGF-1 in the sciatic nerve tissue of DPN rats. Zhang et al believe that oxidative stress is a pathogenic factor causing DPN. They found that increasing the activities of SOD and GSH-PX and reducing the content of MDA can improve lipid peroxidation and inhibit nerve damage in DPN. This is consistent with our research results. However, they also conducted research on the influence of oxidative stress on the apoptotic signaling pathway. They found that oxidative stress can also affect the expression of apoptosis-related factors in treating DPN.[87] These findings provide a direction for our subsequent research.

In the nervous system, although hyperglycemia may be the initiating factor for peripheral nerve injury, there is increasing evidence showing that lipid metabolism disorder is an important factor promoting the formation of DPN in both type 1 and type 2 diabetes.[88] In vitro studies have also found that free fatty acids can directly produce lipotoxic effects on neurons and Schwann cells,[89] and this toxic effect may be mediated through lysosomal dysfunction, which is related to the penetration of cathepsin L into the lysosomal membrane, leading to oxidative stress and mitochondrial damage. A quantitative lipidomics study showed that the content of saturated fatty acids in myelin extracted from the sciatic nerve of diabetic rats was abnormally increased.[90] Our study found that palmityl sphingomyelin, palmitic acid, and full-cis (−6,9,12) -linolenic acid and other lipid metabolites in the model group showed changes. The levels of fatty acids such as stearoyl compounds and linolenic acid in the metabolites of the rats in the administration group were callback. This indicated that YXRJP had a certain role in regulating lipid metabolism. Zhang et al also found that DPN is related to the accumulation of saturated fatty acids. The TCM compound can change the abnormal lipid metabolism of DPN rats. This is consistent with our research results.[91] In addition, we also found that YXRJP not only has a regulatory effect on the stearoyl and phospholipid fatty acids it involved, but also has a regulatory effect on linolenic acid. Linolenic acid is a polyunsaturated fatty acid that can maintain the normal structure and function of nerve membranes. Moreover, research reports have shown that linolenic acid has a good therapeutic effect on patients with DPN.[92]

Arginine is considered to be functional amino acid, which plays important roles in animal metabolism, physiology and various systems (especially maintenance, circulation, reproduction and immune system).[93] Arginine can maintain glucose homeostasis in the body, improve insulin sensitivity, protect islet β cells and inhibit oxidative stress caused by diabetes.[94] First, arginine can stimulate β cells regeneration and increase the area of insulin immunopositive cells, thereby protecting β cells.[95] Secondly, L-arginine can reduce insulin resistance by improving vasodilation mediated by

NO, and can enhance glucokinase activity in rat hepatocytes, thereby improving peripheral and hepatic insulin sensitivity in patients with type 2 diabetes.[96] Long-term oxidative stress and endothelial dysfunction are major sources of risk for diabetes and its complications.[97] Arginine can improve oxidative stress by preventing the downregulation of cellular antioxidants, and this has been demonstrated in different species and cell lines.[98] Our study found that YXRJP may treat DPN by regulating amino acid metabolism such as that of arginine. Studies have also confirmed that the mechanism of TCM in treating DPN is related to amino acid metabolism.[99,100] The difference is that we have enriched the possible related metabolic pathways to include arginine, aspartic acid, glutamic acid and so on. Existing studies have shown that the arginine metabolic pathway plays a key role in the treatment of DPN.[101] Studies have shown that the glutamic acid metabolic pathway and aspartic acid metabolic pathway are closely related to the occurrence of DPN.[102,103]

Besides the metabolic disorders mentioned above, the plasma levels of 2' -deoxycytidine and cytidine in DPN rats were reduced by YXRJP. Nucleotides, which are mainly composed of purines, play multiple roles in human physiology, affecting tissue function, cell integrity, and oxidation. In catabolism, they are converted from monophosphate forms to inosine and guanosine; Purine nucleoside phosphorylase converts them to hypoxanthine and guanine, respectively.[104] Studies have shown increased dephosphorylation of adenine and guanine in diabetic patients compared with healthy subjects. At the same time, the concentrations of adenosine, inosine, guanosine, and hypoxanthine increase, and the conversion rate of nucleotides and hypermetabolism is higher.[105] Studies have confirmed that nucleotides can improve elevated triglycerides and peripheral neuropathy in diabetic animals.[106] Because the pathogenesis of DPN is extremely complex, the metabolites become more complex with the further development of the disease, and the metabolic pathways involved are more diverse. At present, the role of nucleotide metabolism in the metabolic diseases such as diabetes, and in complex metabolism is gradually attracting widespread attention.

We utilized an experimental induction model more consistent with the occurrence and development of human DPN. By using the model rats induced by high-fat diet feeding combined with STZ injection as the study subjects, we found that YXRJP had a favorable therapeutic effect on DPN rats. These findings are expected to provide research directions for the treatment of DPN related diseases with YXRJP and provide scientific basis for further clinical application. It is worth noting that the pathogenesis of DPN is complex, and patients often have other metabolic disorders such as hyperglycemia and hyperlipidemia at the same time. In clinical application, YXRJP may be used in combination with other drugs for the treatment of DPN to meet the needs of complex conditions and achieve the purpose of precise medication. Therefore, the efficacy and safety of YXRJP combination therapy should be investigated before translating these findings to human studies.

In this study, the pharmacological effects of YXRJP on DPN were evaluated and the underlying mechanisms were explored. However, there are some limitations of this study that need to be addressed in future studies. Firstly, the study only investigated the mechanism of the neuroprotective effect of YXRJP. The molecular mechanisms of other signal transduction and metabolic pathways, such as regulating lipid metabolism, ameliorating oxidative stress and reducing inflammatory response, have not been further explored. Secondly, the bioactive component of YXRJP in DPN treatment has not been defined. In the future, we plan to integrate pharmacological effects, compositional analysis, and metabolomics, to further clarify the key bioactive components and mechanism of YXRJP in the treatment of DPN. Finally, although we have already identified the potential biomarkers and pathways of YXRJP in the treatment of DPN, we have not verified them yet. In future studies, we plan to utilize UHPLC-MS technology to detect the changes of metabolites in major tissues (such as the sciatic nerve or blood vessels). Then, we will compare the results with the standards of potential biomarkers (such as 2'-deoxycytidine and Cytidine, etc.) and conduct qualitative and quantitative analyses, so as to determine the impact of YXRJP on the potential biomarkers in major tissues. In addition, we will use the extract of YXRJP to intervene in glucose-induced Schwann cells, detect the changes in the levels of potential biomarkers in the cells and culture medium, and simultaneously detect the changes in the activities of key enzymes related to potential pathways (such as arginase) as well as the amounts of products generated (such as arginine and ornithine, etc)., so as to verify whether YXRJP treats DPN through potential pathways.

Conclusion

In summary, our study analyzed the possible mechanism of action of YXRJP in the treatment of DPN from the perspective of "multi-component, multi-target and multi-pathway" by network pharmacology method. We further conducted experimental verification using animal models, and explored the potential mechanism of YXRJP in treating DPN. Our research found that YXRJP may treat DPN through core targets such as TP53, JUN, MAPK3, NF-KB1 and AKT1, as well as TNF signaling pathway and TLR signaling pathway. Further animal experiments showed that YXRJP could increase the body weight, improve the abnormal response to heat pain, reduce TG content, improve the MNCV of sciatic nerve, and improve the ultrastructural damage of sciatic nerve tissue in DPN rats, which may be related to enhancing the activities of SOD and GSH-PX, reducing the content of MDA, inhibiting the expression of inflammation-related protein AKT, promoting the expression of neuroprotection-related protein IGF-1, affecting amino acid, lipid and nucleotide metabolism. This study reflects the characteristics of YXRJP in treating DPN. The results show that YXRJP is a potential drug for the treatment of DPN, providing a theoretical basis for the clinical application of YXRJP.

Abbreviations

YXRJP, Yangxuerongjin pill; DPN, diabetic peripheral neuropathy; IDF, International Diabetes Federation; WHO, World Health Organization TLR, Toll-like receptor; TNF, tumor necrosis factor; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; OB, oral bioavailability; DL, drug similarity; PPI, protein-protein interactions; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; MF, molecular function; CC, cellular component; OMIM, Online Mendelian Inheritance in Man; SD, Sprague Dawley; SPF, specific pathogen free; YXRJP-H, the high dose of YXRJP group; YXRJP-M, the middle dose of YXRJP group; YXRJP-L, the low dose of YXRJP group; STZ, streptozotocin; TWL, thermal withdrawal latency; MNCV, Motor Nerve Conduction Velocity; TG, triglyceride; GLU, glucose; Gpo-pap, Glycerol phosphate oxidase-phenol aminoantipyrine; GSH-PX, glutathione peroxidase; T-AOC, total antioxidant capacity; SOD, superoxide dismutase; MDA, malondialdehyde; IOD, integrated optical density; NC, Nitrocellulose; IGF-1, Insulin-like growth factor 1; AKT, protein kinase B; UHPLC, ultra-high performance liquid chromatography; HILIC, Hydrophilic Interaction Liquid Chromatography; ESI, electrospray ionization; Gas1, atomizing gas auxiliary heating gas 1; Gas2, auxiliary heating gas 2; CUR, curtain gas; ISVF, ion-source voltage; DDA, data dependent acquisition; DP, declustering potential; PCA, Principal Component Analysis; OPLS-DA, Orthogonal Partial Least Squares-Discriminant Analysis; VIP, variable important in projection; LSD, least significant difference; TIC, total ion current map; TCM, traditional Chinese medicine; ANOVA, analysis of variance; AKT1, Serine/threonine protein kinase 1; MBP, myelin basic protein; rhTNFR:Fc, recombinant human TNF-α receptor II-Fc fusion protein.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics Statements

All datasets in this study were downloaded from public databases. The current research follows database access policies and publication guidelines. According to item 1 and 2 of Article 32 of "the Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects", this study is exempt from ethical review and approval.

The experimental scheme was approved by Experimental Animal Ethics Committee of Beijing Tongrentang Research Institute (the ethical approval number: YJY-2021-031001). The experimental process is strictly in accordance with the China's guidelines for the ethical review of laboratory animal welfare.

Acknowledgments

This work was supported by the Beijing Tongrentang Technology Development Co., Ltd. Pharmaceutical factory.

Disclosure

The authors declare no conflicts of interest in this work.

References

1. Yang XY, Zhang L, Chen X. Research progress of diabetic peripheral neuropathy mechanisms in type 2 diabetes. *Chinese Pharm Bulletin*. 2016;32(5):598–602.
2. Sun H, Saeedi P, Karuranga S, et al. IDF Diabetes Atlas: global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract*. 2022;183:109119. doi:10.1016/j.diabres.2021.109119
3. Tang F, Zhao FK, Jiang Y, Zhang T, Wang BF. Global hotspots and trends in diabetic peripheral neuropathy research from 2011 to 2023. *Medicine*. 2024;103(32):e39295. doi:10.1097/MD.0000000000039295
4. Tavakoli M, Klingelhöfer D, Fadavi H, Groneberg DA. The landscape of global research on diabetic neuropathy. *Front Endocrinol*. 2023;14:1220896. doi:10.3389/fendo.2023.1220896
5. Singh U, Sharma R, Kumar R. An Overview on Diabetic Neuropathy. *Current Diabetes Rev*. 2024;20. doi:10.2174/0115733998295741240606104106
6. Liu WJ. Influencing factors of abnormal peripheral nerve conduction velocity in patients with type 2 diabetes mellitus. *Clin Res Pra*. 2024;9(08):23–26.
7. Kim SS, Won JC, Kwon HS, et al. Prevalence and clinical implications of painful diabetic peripheral neuropathy in type 2 diabetes: results from a nationwide hospital-based study of diabetic neuropathy in Korea. *Diabetes Res Clin Pract*. 2014;103(3):522–529. doi:10.1016/j.diabres.2013.12.003
8. Carmichael J, Fadavi H, Ishibashi F, Shore AC, Tavakoli M. Advances in screening, early diagnosis and accurate staging of diabetic neuropathy. *Front Endocrinol*. 2021;12:671257. doi:10.3389/fendo.2021.671257
9. Zhang P, Wang ZM, Sui M, Zhou CL, Li C. Clinical observation on the Danteng Tongmai prescription on peripheral neuropathy of type 2 diabetes mellitus. *Clin J Chinese Med*. 2024;16(02):144–148.
10. Yang K, Wang Y, Li YW, et al. Progress in the treatment of diabetic peripheral neuropathy. *Biomed. Pharmacother.* 2022;148:112717. doi:10.1016/j.biopha.2022.112717
11. Lin HJ, Liu YF, Zhan GL. Efficacy of Yiqi Wenyang Huoxue Tongluo Prescription Combined with Acupuncture on Type 2 Diabetic Peripheral Neuropathy OF Qi Deficiency and Blood Stasis and Its Effect on Oxidative Stress. *World J Integrated Tradi Western Medi*. 2022;17(02):354–358+363.
12. Zhang D, Zheng SQ. Discussion on the prescription rules of tongluo method in the treatment of diabetic peripheral neuropathy. *Asia-Pacific Tradit Med*. 2021;17(05):158–161.
13. Chinese Pharmacopoeia Commission. *Pharmacopoeia of the People's Republic of China 2020*. China Medical Science and Technology Press; 2020.
14. Su HN, Wang DJ, Wang SL. Report on the comprehensive clinical evaluation of Yangxue Rongjin pills. *China Practical Med*. 2022;17(24):168–173.
15. Gao S, Yang FM. Effective Evaluation of Xingnao Tongdu Acupuncture and Yangxue Rongjin Pills in the Treatment of Spasticity after Stroke. *Chi J Integ Med Cardio-Cerebrovar Dis*. 2017;15(23):2963–2965.
16. Zhang XY, Wang HT, Zhang Y, et al. Danggui Sini decoction for treating diabetic peripheral neuropathy: a protocol of systematic review and meta-analysis of randomized controlled trials. *Medicine*. 2020;99(21):e20482. doi:10.1097/MD.0000000000020482
17. Shi YQ, Li HR, Lin YG, Wang SF, Shen GF. Effective constituents and protective effect of Mudan granules against Schwann cell injury. *J Ethnopharmacol*. 2024;323:117692. doi:10.1016/j.jep.2023.117692
18. Zhao JD, Li Y, Xin L, et al. Clinical features and rules of Chinese herbal medicine in diabetic peripheral neuropathy patients. *Evid Based Complement Alternat Med*. 2020;2020. doi:10.1155/2020/5795264
19. Yang XH, Deng YY. The Impact of Xu Duan Tong Luo Pian on Nerve Conduction Velocity of Experimental Diabetic Rats. *Chinese J Phar*. 2010;7(10):577.
20. Hou YW, Li YM, Xu YK, Xiao ZW. Discussion on the molecular mechanism of spatholive-mulberry parasitism in the treatment of diabetic peripheral neuropathy based on network pharmacology and molecular docking. *Chin Med Herald*. 2021;18(26):24–29+198.
21. Al-Rejaie SS, Aleisa AM, Abuohashish HM, et al. Naringenin neutralises oxidative stress and nerve growth factor discrepancy in experimental diabetic neuropathy. *Neurological Res*. 2015;37(10):924–933. doi:10.1179/1743132815Y.0000000079
22. Ghazaee H, Raouf Sheibani A, Mahdian H, Gholami S, Askari VR, Baradaran Rahimi V. Ellagic acid as potential therapeutic compound for diabetes and its complications: a systematic review from bench to bed. *Naunyn-Schmiedeberg's Arch Pharmacol*. 2024;1–22.
23. Tang C, Han R, Wu JL, Fang F. Effects of baicalin capsules combined with α-lipoic acid on nerve conduction velocity, oxidative stress and inflammatory injury in patients with diabetic peripheral neuropathy. *Am J Transl Res*. 2021;13(4):2774.
24. Stavniichuk R, Drel VR, Shevalye H, et al. Baicalein alleviates diabetic peripheral neuropathy through inhibition of oxidative–nitrosative stress and p38 MAPK activation. *Exp Neurol*. 2011;230(1):106–113. doi:10.1016/j.expneurol.2011.04.002
25. Li M, Li Q, Zhao QS, Zhang JC, Lin J. Luteolin improves the impaired nerve functions in diabetic neuropathy: behavioral and biochemical evidences. *Int J Clin Exp Pathol*. 2015;8(9):10112.
26. Zhang Q, Song W, Zhao BJ, et al. Quercetin attenuates diabetic peripheral neuropathy by correcting mitochondrial abnormality via activation of AMPK/PGC-1α pathway in vivo and in vitro. *Front Neurosci*. 2021;15:636172. doi:10.3389/fnins.2021.636172
27. Kishore L, Kaur N, Singh R. Effect of Kaempferol isolated from seeds of Eruca sativa on changes of pain sensitivity in Streptozotocin-induced diabetic neuropathy. *Inflammopharmacology*. 2018;26:993–1003. doi:10.1007/s10787-017-0416-2
28. Zhang XD, Yue F, Zhang ZC, et al. Neuroprotective effect of YangxueRongjin pill on taxol-induced peripheral nerve injury in rats. *Chinese Tradit Patent Med*. 2024;46(06):2069–2073.
29. Ma YQ, Chen J, Huang XG, Liu Y. The efficacy and safety of mecobalamin combined with Chinese medicine injections in the treatment of diabetic peripheral neuropathy: a systematic review and Bayesian network meta-analysis of randomized controlled trials. *Front Pharmacol*. 2022;13:957483. doi:10.3389/fphar.2022.957483
30. Han YJ, Wang M, Shen J, et al. Differential efficacy of methylcobalamin and alpha-lipoic acid treatment on symptoms of diabetic peripheral neuropathy. *Minerva Endocrinologica*. 2016;43(1):11–18. doi:10.23736/S0391-1977.16.02505-0

31. Sawangjit R, Thongphui S, Chaichompu W, Phumart P. Efficacy and safety of mecobalamin on peripheral neuropathy: a systematic review and meta-analysis of randomized controlled trials. *J Altern Complementary Med*. 2020;26(12):1117–1129. doi:10.1089/acm.2020.0068
32. Ru JL, Li P, Wang JN, et al. TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. *J Cheminform*. 2014;6 (1):13. doi:10.1186/1758-2946-6-13
33. Stelzer G, Rosen N, Plaschkes I, et al. The GeneCards Suite: from Gene Data Mining to Disease Genome Sequence Analyses. *Curr protoc bioinf*. 2016;54. doi:10.1002/cpbi.5
34. Amberger JS, Bocchini CA, Schiettecatte F, Scott AF, Hamosh A. OMIM.org: online Mendelian Inheritance in Man (OMIM), an Online catalog of human genes and genetic disorders. *Nucleic Acids Res*. 2014;43(D1). doi:10.1093/nar/gku1205
35. Piero J, Ramírez-Anguita JM, Saüch-Pitarch J, Ronzano F, Furlong LI. The DisGeNET knowledge platform for disease genomics: 2019 update. *Nucleic Acids Res*. 2019;48(D1).
36. Wishart DS, Feunang YD, Guo AC, et al. DrugBank 5.0: a major update to the DrugBank database for 2018. *Oxford University Press*. 2018.
37. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res*. 2018.
38. Sherman BT, Ming H, Ju Q, et al.DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acids Res*. 2022;(W1):W1. doi:10.1093/nar/gkac525
39. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*. 2009;4(1):44. doi:10.1038/nprot.2008.211
40. Allgoewer A, Mayer B. Sample size estimation for pilot animal experiments by using a Markov Chain Monte Carlo approach. *Altern Lab Anim*. 2017;45(2):83–90. doi:10.1177/026119291704500201
41. Xu SY, Bian RL, Chen X. *Experimental Methodology of Pharmacology*. People's Medical Publishing House Co.LTD; 2002.
42. Chen Q, Zhang BL. *Methodology for Pharmacodynamic Research in Traditional Chinese Medicine*. People's Medical Publishing House Co. LTD; 2016.
43. Sun ZY, Liu YD, Zhao YH, Xu YH. Animal Models of Type 2 Diabetes Complications: a Review. *Endocrine Res*. 2024;49(1):46–58. doi:10.1080/07435800.2023.2278049
44. Furman BL. Streptozotocin-induced diabetic models in mice and rats. *Current Protocols*. 2021;1(4):e78. doi:10.1002/cpz1.78
45. Choi JS, de Haan JB, Sharma A. Animal models of diabetes-associated vascular diseases: an update on available models and experimental analysis. *Br.J Pharmacol*. 2022;179(5):748–769. doi:10.1111/bph.15591
46. Ota A, Kakehashi A, Toyoda F, et al. Effects of long-term treatment with ranirestat, a potent aldose reductase inhibitor, on diabetic cataract and neuropathy in spontaneously diabetic torii rats. *Journal of Diabetes Research*. 2013;2013(1):175901. doi:10.1155/2013/175901
47. Zhang EL, Li MQ, Zhao JJ, Dong YX, Yang XQ, Huang JB. Traditional Chinese medicine Yisui Tongjing relieved neural severity in experimental autoimmune neuritis rat model. *Neuropsychiatr Dis Treat*. 2016;2481–2487. doi:10.2147/NDT.S110813
48. Nonaka K, Akiyama J, Une S. Exercise May Increase Oxidative Stress in the Sciatic Nerve in Streptozotocin-Induced Diabetic Rats. *Medicina*. 2024;60(3):480. doi:10.3390/medicina60030480
49. Liu YM, Dong RQ, Zhang CY, et al. Therapeutic effects of nerve leachate-treated adipose-derived mesenchymal stem cells on rat sciatic nerve injury. *Exp Ther Med*. 2020;19(1):223–231. doi:10.3892/etm.2019.8203
50. Ebrahimi HA, Jafari M, Khanjani N, Moradi N. Evaluation of Date Extract on Nerve Conduction Velocity in Male Rats. *Basic Clin Neurosci*. 2022;13(3):385. doi:10.32598/bcn.2021.878.2
51. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*. 2012;9(7):671–675. doi:10.1038/nmeth.2089
52. *R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*. Austria: Vienna; 2024.
53. DH O, JC D, AP W AD. FSA: simple Fisheries Stock Assessment Methods. R package version 0.9.5; 2023.
54. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res*. 2009;37(1):1–13. doi:10.1093/nar/gkn923
55. Moradi E, Marttinen M, Häkkinen T, Hiltunen M, Nykter M. Supervised pathway analysis of blood gene expression profiles in Alzheimer's disease. *Neurobiol Aging*. 2019;84:98–108. doi:10.1016/j.neurobiolaging.2019.07.004
56. Iqbal Z, Azmi S, Yadav R, et al. Diabetic peripheral neuropathy: epidemiology, diagnosis, and pharmacotherapy. *Clin. Ther.* 2018;40 (6):828–849. doi:10.1016/j.clinthera.2018.04.001
57. Zhu JX, Hu ZY, Luo YF, et al. Diabetic peripheral neuropathy: pathogenetic mechanisms and treatment. *Front Endocrinol*. 2024;14:1265372. doi:10.3389/fendo.2023.1265372
58. Tay JS, Kim YJ. Efficacy of moxibustion in diabetes peripheral neuropathy. *Medicine*. 2021;100(49):e28173. doi:10.1097/MD.0000000000028173
59. Piao YL, Liang XC. Chinese medicine in diabetic peripheral neuropathy: experimental research on nerve repair and regeneration. *Evidence-Based Complem Alternative Med*. 2012;2012(1):191632. doi:10.1155/2012/191632
60. Wang JS, Yin HJ, Chen KJ. Research progress on mechanism of activating blood circulation to remove stasis treatment for diabetic angiopathy. *Chinese J Integrated Traditional and Western Medicine*. 2014;34(11):1397–1400.
61. He LS, Wang H, Gu CJ, He XH, Zhao LH, Tong XL. Administration of traditional Chinese blood circulation activating drugs for microvascular complications in patients with type 2 diabetes mellitus. *Journal of Diabetes Research*. 2016;2016(1):1081657. doi:10.1155/2016/1081657
62. Pang GM, Yan Y, Zhu P, et al. Clinic draft specification of traditional Chinese medicine about diabetic peripheral neuropathy. *China J Tradition Chinese Med Pharm*. 2010;25(2):260–264.
63. Zhang YN, Wu XL, Yao WH, Ni YD, Ding XS. Advances of traditional Chinese medicine preclinical mechanisms and clinical studies on diabetic peripheral neuropathy. *Pharm Biol*. 2024;62(1):544–561. doi:10.1080/13880209.2024.2369301
64. Tong XL, Dong L, Chen L, Zhen Z. Treatment of diabetes using traditional Chinese medicine: past, present and future. *Am J Chin Med*. 2012;40 (05):877–886. doi:10.1142/S0192415X12500656
65. Tian JX, Lian FM, Yu XT, et al. The efficacy and safety of Chinese herbal decoction in type 2 diabetes: a 5-year retrospective study. *Evidence-Based Complem Alternative Med*. 2016;2016(1):5473015. doi:10.1155/2016/5473015

66. Li MD, Su H, Xiang YY, Zhang JY, Zhao JJ, Lin L. Features analysis on traditional Chinese medicine syndromes in patients with diabetic peripheral neuropathy. *J Traditional Chin Med*. 2013;33(5):637–641. doi:10.1016/S0254-6272(14)60034-0
67. Qiao YL, Guo GY, Zhao JH. Comparative study of two integrated traditional Chinese and Western medicine treatment methods on treatment compliance of patients with diabetic peripheral neuropathy. *Folia Neuropathologica*. 2022;60(3):324–328. doi:10.5114/fn.2022.117523
68. Kim S, Maynard JC, Strickland A, Burlingame AL, Milbrandt J. Schwann cell O-GlcNAcylation promotes peripheral nerve remyelination via attenuation of the AP-1 transcription factor JUN. *Proceedings of the National Academy of Sciences*. 2018;115:8019–8024.
69. Zhou CL, Li F, Wu XW, et al. Overexpression of miRNA-433-5p protects acute spinal cord injury through activating MAPK1. *Eur Rev Med Pharmacol Sci*. 2020;24(6).
70. Luo L, Zhou WH, Cai JJ, et al. Gene expression profiling identifies downregulation of the neurotrophin-MAPK signaling pathway in female diabetic peripheral neuropathy patients. *Journal of Diabetes Research*. 2017;2017(1):8103904. doi:10.1155/2017/8103904
71. Chen L, Gong HY, Xu L. PVT1 protects diabetic peripheral neuropathy via PI3K/AKT pathway. *Eur Rev Med Pharmacol Sci*. 2018;22(20).
72. Dandawate S, Williams L, Joshee N, et al. Scutellaria extract and wogonin inhibit tumor-mediated induction of T reg cells via inhibition of TGF-β1 activity. *Cancer Immunol Imunother*. 2012;61:701–711. doi:10.1007/s00262-011-1130-3
73. Chen SP, Zhou YQ, Liu DQ, et al. PI3K/Akt pathway: a potential therapeutic target for chronic pain. *Curr Pharm Des*. 2017;23(12):1860–1868. doi:10.2174/1381612823666170210150147
74. Pan BB, Ding HJ, Cheng ZG, Song ZB, Xiao D, Guo QL. Vascular endothelial growth factor antibody attenuates diabetic peripheral neuropathic pain in rats. *Zhong Nan da Xue Xue Bao Yi Xue Ban*. 2018;43(10):1097–1102. doi:10.11817/j.issn.1672-7347.2018.10.009
75. Wang XK, Ye KH, Lv YQ, et al. Ameliorative effect and underlying mechanisms of total triterpenoids from Psidium guajava Linn (myrtaceae) leaf on high-fat streptozotocin-induced diabetic peripheral neuropathy in rats. *Trop J Pharm Res*. 2016;15(2):327–333. doi:10.4314/tjpr.v15i2.15
76. Liu K, Yang YC, Zhou F, Xiao YD, Shi LW. Inhibition of PI3K/AKT/mTOR signaling pathway promotes autophagy and relieves hyperalgesia in diabetic rats. *Neuroreport*. 2020;31(9):644–649. doi:10.1097/WNR.0000000000001461
77. Li DL, He T, Chen BT, Yilzera A, Ma XL. Study on the mechanism of different parts of Morus alba L. in preventing and treating diabetes based on network pharmacology-molecular docking. *Nat Prod Res Dev*. 2021.
78. Shi XH, Chen YH, Nadeem L, Xu GX. Beneficial effect of TNF-α inhibition on diabetic peripheral neuropathy. *J Neuroinflammation*. 2013;10:1–9. doi:10.1186/1742-2094-10-69
79. Fang HL, Wu YF, Huang XH, et al. Toll-like receptor 4 (TLR4) is essential for Hsp70-like protein 1 (HSP70L1) to activate dendritic cells and induce Th1 response. *J Biol Chem*. 2011;286(35):30393–30400. doi:10.1074/jbc.M111.266528
80. Zhou HG, Chen HB, Wu MH. Viewing from the toll-like receptor/nuclear factor-kappaB signaling pathway to explore the immunomodulatory mechanism of Chinese drugs. *Zhongguo Zhong xi yi jie he za zhi Zhongguo Zhongxiyi Jiehe Zazhi*. 2010;30(8):884–888.
81. Fan BY, Li C, Szalad A, et al. Mesenchymal stromal cell-derived exosomes ameliorate peripheral neuropathy in a mouse model of diabetes. *Diabetologia*. 2020;63:431–443. doi:10.1007/s00125-019-05043-0
82. Wei W, Mao H, Zhao S. Assessment of sensory current threshold in the diagnosis of type 2 diabetic peripheral neuropathy clinical application of current perception threshold in the diagnosis of diabetic peripheral neuropathy. *Chin J Diab*. 2018;50–54.
83. Yang JZ, Wei YX, Zhao T, et al. Magnolol effectively ameliorates diabetic peripheral neuropathy in mice. *Phytomedicine*. 2022;107:154434. doi:10.1016/j.phymed.2022.154434
84. Huang YW. Diagnostic Significance of Nerve Conduction Velocity Study in Diabetic Peripheral Neuropathy. *Neural Injury Funct Reconstr*. 2006;1(03):159–160.
85. Peng HY, Gong YY. Analysis of the effect of probucol-mecobalamin tablets combination on oxidative stress in patients with diabetic peripheral neuropathy. *Neurosci lett*. 2021;741:135484. doi:10.1016/j.neulet.2020.135484
86. Song W, Jiang W, Wang C, et al. Jinmaitong, a Traditional Chinese Compound Prescription, Ameliorates the Streptozocin-Induced Diabetic Peripheral Neuropathy Rats by Increasing Sciatic Nerve IGF-1 and IGF-1R Expression. *Front Pharmacol*. 2019;10:255. doi:10.3389/fphar.2019.00255
87. Zhang YZ, Zhou ZC, Song CY, Chen X. The protective effect and mechanism of dexmedetomidine on diabetic peripheral neuropathy in rats. *Front Pharmacol*. 2020;11:1139. doi:10.3389/fphar.2020.01139
88. Smith AG, Singleton JR. Obesity and hyperlipidemia are risk factors for early diabetic neuropathy. *J diabet complicat*. 2013;27(5):436–442. doi:10.1016/j.jdiacomp.2013.04.003
89. McCall KD, Holliday D, Dickerson E, et al. Phenylmethimazole blocks palmitate-mediated induction of inflammatory cytokine pathways in 3T3L1 adipocytes and RAW 264.7 macrophages. *J Endocrinol*. 2010;207(3):343. doi:10.1677/JOE-09-0370
90. Mitro N, Cermenati G, Brioschi E, et al. Neuroactive steroid treatment modulates myelin lipid profile in diabetic peripheral neuropathy. *J Steroid Biochem mol Biol*. 2014;143:115–121. doi:10.1016/j.jsbmb.2014.02.015
91. Zhang Q, Song W, Liang XC, Xie J, Chen XT. A Metabolic Insight Into the Neuroprotective Effect of Jin-Mai-Tong (JMT) Decoction on Diabetic Rats With Peripheral Neuropathy Using Untargeted Metabolomics Strategy. *Frontiers in Pharmacology*. 2020;11.
92. Won JC, Kwon HS, Moon SS, et al. γ-Linolenic Acid versus α-Lipoic Acid for Treating Painful Diabetic Neuropathy in Adults: a 12-Week, Double-Placebo, Randomized, Noninferiority Trial. *Korean Diab Associ*. 2020.
93. Huang C, Song PX, Fan PX, Hou CL, Thacker P, Ma X. Dietary sodium butyrate decreases postweaning diarrhea by modulating intestinal permeability and changing the bacterial communities in weaned piglets. *J Nutr*. 2015;145(12):2774–2780. doi:10.3945/jn.115.217406
94. Russell RR III, Bergeron R, Shulman GI, Young LH. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol Heart Circ Physiol*. 1999;277(2):H643–H649. doi:10.1152/ajpheart.1999.277.2.H643
95. Xue J, Davidson PM, Zhong QX. Thymol nanoemulsified by whey protein-maltodextrin conjugates: the enhanced emulsifying capacity and antilisterial properties in milk by propylene glycol. *Journal of Agricultural and Food Chemistry*. 2013;61(51):12720–12726. doi:10.1021/jf4043437
96. Matsumoto T, Kobayashi T, Ishida K, Taguchi K, Kamata K. Enhancement of mesenteric artery contraction to 5-HT depends on Rho kinase and Src kinase pathways in the ob/ob mouse model of type 2 diabetes. *Br. J. Pharmacol.* 2010;160(5):1092–1104. doi:10.1111/j.1476-5381.2010.00753.x

97. Gohir W, Whelan FJ, Surette MG, Moore C, Schertzer JD, Sloboda DM. Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet. *Gut Microbes*. 2015;6(5):310–320. doi:10.1080/19490976.2015.1086056
98. Orlando G, Wolf G, Engelmann M. Role of neuronal nitric oxide synthase in the regulation of the neuroendocrine stress response in rodents: insights from mutant mice. *Amino Acids*. 2008;35:17–27. doi:10.1007/s00726-007-0630-0
99. Zhang KH, Peng P, Huang JH, et al. Integrating plasma metabolomics and gut microbiome to reveal the mechanisms of Huangqi Guizhi Wuwu Decoction intervene diabetic peripheral neuropathy. *J Ethnopharmacol*. 2024;319:117301. doi:10.1016/j.jep.2023.117301
100. Li YF, Yao WJ, Gao YB. Effects of Tang Luo Ning on diabetic peripheral neuropathy in rats revealed by LC–MS metabolomics approach. *Biomed. Chromatogr*. 2022;(7):36.
101. Liu Q, Chen YF, Wang B, et al. Arginine Biosynthesis Pathway Found to Play a Key Role in the Neuroprotective Effect of Liu-Wei-Luo-Bi (LWLB) Granules in Diabetic db/db Mice with Peripheral Neuropathy Using an Untargeted Metabolomics Strategy. *Diab Metab Synd Obesity*;2023. 4065–4080. doi:10.2147/DMSO.S423388
102. Hussain N, Adrian TE. Diabetic neuropathy: update on pathophysiological mechanism and the possible involvement of glutamate pathways. *Current Diabetes Rev*. 2017;13(5):488–497. doi:10.2174/1573399812666160624122605
103. Shao MM, Xiang HJ, Lu H, et al. Candidate metabolite markers of peripheral neuropathy in Chinese patients with type 2 diabetes. *Am J Transl Res*. 2022;14(8):5420.
104. Papandreou C, Li J, Liang LM, et al. Metabolites related to purine catabolism and risk of type 2 diabetes incidence; modifying effects of the TCF7L2-rs7903146 polymorphism. *Sci Rep*. 2019;9(1):2892. doi:10.1038/s41598-019-39441-6
105. Dudzinska W. Purine nucleotides and their metabolites in patients with type 1 and 2 diabetes mellitus. *J Biomed Sci Eng*. 2014;2014.
106. Chandrasekaran K, Najimi N, Sagi AR, et al. NAD+ precursors repair mitochondrial function in diabetes and prevent experimental diabetic neuropathy. *Int J mol Sci*. 2022;23(9):4887. doi:10.3390/ijms23094887