Diabetes, Metabolic Syndrome and Obesity Dovepress

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Network Pharmacology-Based Strategy to Explore the Effect and Mechanism of Zhizhu Granule Improving Glucose-Lipid Metabolism in Rats with Metabolic Syndrome

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Objective: To explore the mechanism of the traditional Chinese medicine (TCM), Zhizhu granule (ZZG), in treating metabolic syndrome (MS) based on network pharmacology and pharmacodynamic experiment.

Materials and Methods: Network pharmacology combined with a pharmacodynamic experiment was used to elucidate the therapeutic mechanism of ZZG in MS. Serum samples were collected from rats with MS, induced by a high-sugar-fat-salt diet (HSFSD) combined with streptozotocin (STZ), to measure the levels of biochemical markers. The glucose (GLU), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were detected. The liver tissue of rats was used for histological examination and Western blot analysis.

Results: Network pharmacology analysis generated 69 drug-disease common targets and 10 hub genes closely related to ZZG against MS. KEGG pathway analysis revealed that the PI3K/AKT signaling pathway was the most potential pathway, which took part in the therapeutic mechanisms. In the animal experiments section, the therapeutic effect of ZZG on MS and the therapeutic pathway of ZZG on MS were verified. ZZG could significantly decrease the body weight, TC, TG, LDL-C and GLU levels in MS rats (all $p<0.01$), alleviate hepatocyte steatosis and decrease liver lipid droplet deposition. Western blot analysis indicated that compared with the model group, the expression levels of PI3K, AKT, and IRS-1 protein were significantly increased (all $p<0.05$), and the FOXO-1 was significantly decreased (all $p<0.05$) in the ZZG group.

Conclusion: ZZG can improve glucose-lipid metabolism disorder in rats with metabolic syndrome. The reported results provide experimental evidence for ZZG in the treatment of MS.

Keywords: metabolic syndrome, network pharmacology, Zhizhu granule, pharmacodynamic experiment, PI3K/AKT pathway

Introduction

Metabolic syndrome (MS) is a complex syndrome that includes obesity, hypertension, hyperglycemia, and dyslipidemia. In 2005, the International Diabetes Foundation (IDF) published new criteria for metabolic syndrome. The content of the criteria includes: a. central obesity (waist circumference): ≥94 cm(M), ≥80cm(F); b. obesity, plus two of the four criteria below; c. fasting glucose ≥100mg/dl; d. TG ≥150mg/dl or Rx; e. HDL cholesterol: <40mg/dl(M), <50mg/dl(F), or Rx; f. >130mmHg systolic or >85mmHg diastolic or Rx.[1] In addition to a healthy lifestyle, Butyrate, Probiotics, Asymmetric Dimethylarginine, Curcumin, Statins and other drugs can be truly used as a coadjuvant therapy for this pathology.[2]

According to existing reports, the prevalence of MS in adults worldwide is about 20%-25%,[3] reaching up to 34.7% in the United States, and the prevalence tends to increase with age, affecting ever younger individuals.[4] It may increase the

Received: 8 May 2024
Accepted: 26 September 2024
Published: 18 October 2024

risk of cardiovascular diseases and type 2 diabetes mellitus approximately 2-fold and 5-fold or more, respectively.[5] In addition, hyperuricemia, thyroid dysfunction and non-alcoholic fatty liver disease are significantly associated with the development and severity of the metabolic syndrome.[6–8] Also, several studies support a possible negative impact of MS on male reproductive potential, reporting a negative association between MS and sperm parameters, testosterone and follicle-stimulating hormone levels.[9] MS is becoming a serious clinical and public health challenge, requiring greater attention. Traditional Chinese medicine (TCM) hold that the occurrence of MS is caused by the dysfunction of the kidney, liver and spleen, and is related to drinking, sitting for a long time, eating fat and sweet taste and insomnia, so it produces phlegm dampness, blood stasis, Qi deficiency, Yang deficiency, Yin deficiency and other syndromes. The treatment needs to apply Chinese herbs to regulate the function of the kidney, liver and spleen, dispel phlegm and remove dampness, promote blood circulation and remove blood stasis, and supplement Yin, Yang and Qi of deficiency.

TCM is a treasure of Chinese civilization that has a significant role in ancient and modern China. It is characterized by multi-component, multitarget, and multi-pathway management, which is especially suitable for complex conditions with multiple symptoms such as MS. Zhizhu Granule (ZZG) is a Chinese patent medicine commonly used in clinical practice, which comprises three herbs, ie, *Fructus Aurantii Immaturus* [Rutaceae] (Chinese name: Zhishi), *Atractylodes Macrocephala Koidz* [Asteraceae] (Chinese name: Baizhu), and *Nelumbo nucifera Gaertn* [Nelumbonaceae] (Chinese name: Heye). The names of all the herbs have been certified (http://mpns.kew.org). Its primary indication in Chinese medicine is to strengthen the spleen, aid digestion, promote the flow of qi, and dissipate dampness. ZZG are usually used to treat functional dyspepsia and gastro-esophageal reflux in clinical practice.[10,11] Also, its main ingredient clinically used to treat hypertension, hyperglycemia, and dyslipidemia.[12–14]

Modern pharmacology found that Zhishi can reduce the production of inflammatory mediators and block the inflammatory process by inhibiting the MAPK signaling pathway and NF-κB signaling pathway. It can also regulate metabolic disorders by affecting amino acid, glucose, and lipid metabolism.[15] Baizhu exerts antibacterial and anti-inflammatory physiological activities and regulates lipid metabolism.[16] The alkaloids in Heye can enhance AMPK phosphorylation and glucose transporter 4 (GLUT4) expression by activating the AMPK signaling pathway, thereby increasing glucose uptake and reducing lipid accumulation. Baizhu can improve lipid distribution in type 2 diabetic mice and lower total cholesterol, triglyceride, and low-density lipoprotein levels in the liver by activating PPAR/PGC1α pathway.[17] Therefore, ZZG can improve metabolic disorders to a certain extent, especially glucose and lipid metabolism, and is often used in traditional Chinese medicine (TCM) prescriptions for treating MS. However, there are only a few studies on the therapy of MS by ZZG, so the molecular mechanisms of the therapy remain unclear due to the complexity of the interactions among components, targets, and the disease. Both the efficacy of TCM and modern pharmacology suggest that ZZG has a certain effect on the treatment of MS.

Network pharmacology is an innovative method used to clarify the synergistic effect and underlying mechanism of component-target network and target-disease network, which is essential in revealing the molecular mechanisms underlying drug treatment diseases, especially to reveal the mechanism of TCM.[18] It can analyze the links among drugs, targets, and diseases by those biological networks. This approach effectively bridges the gap between modern medicine and TCM, and it greatly facilitates studies into the synergistic actions of TCM.[19] It can be widely used to study TCM for efficacy research, mechanism elucidation, target prediction, safety evaluation, drug repurposing, and drug design. The development and application of network pharmacology has promoted the safety, efficacy, and mechanism investigations of TCM, which then reinforces the credibility and popularity of TCMs.[20]

ZZG, whose efficacy has been tested in clinical practice, have great therapeutic value and represent an excellent resource for drug discovery. However, TCM usually treats complex diseases with a "multi-component, multi-target, multi-pathway" paradigm. New ideas and methods are urgently needed to explain the complex interactions between ZZG and MS. Network pharmacology can help us to uncover and visualize the underlying interaction networks of ZZG against MS. This study used a network pharmacology method to predict whether ZZG could act on MS targets. Then, the treatment effects and possible mechanisms were verified using a rat MS model. To the best of our knowledge, this is the first systematic research on the effects and mechanisms of ZZG against MS, which further clarifies the mechanism of multitarget regulation of MS in Chinese medicine and elucidates the screening of core compounds. These findings also offer an experimental basis for utilizing ZZG as a candidate drug for MS, thus facilitating the discovery of novel effective

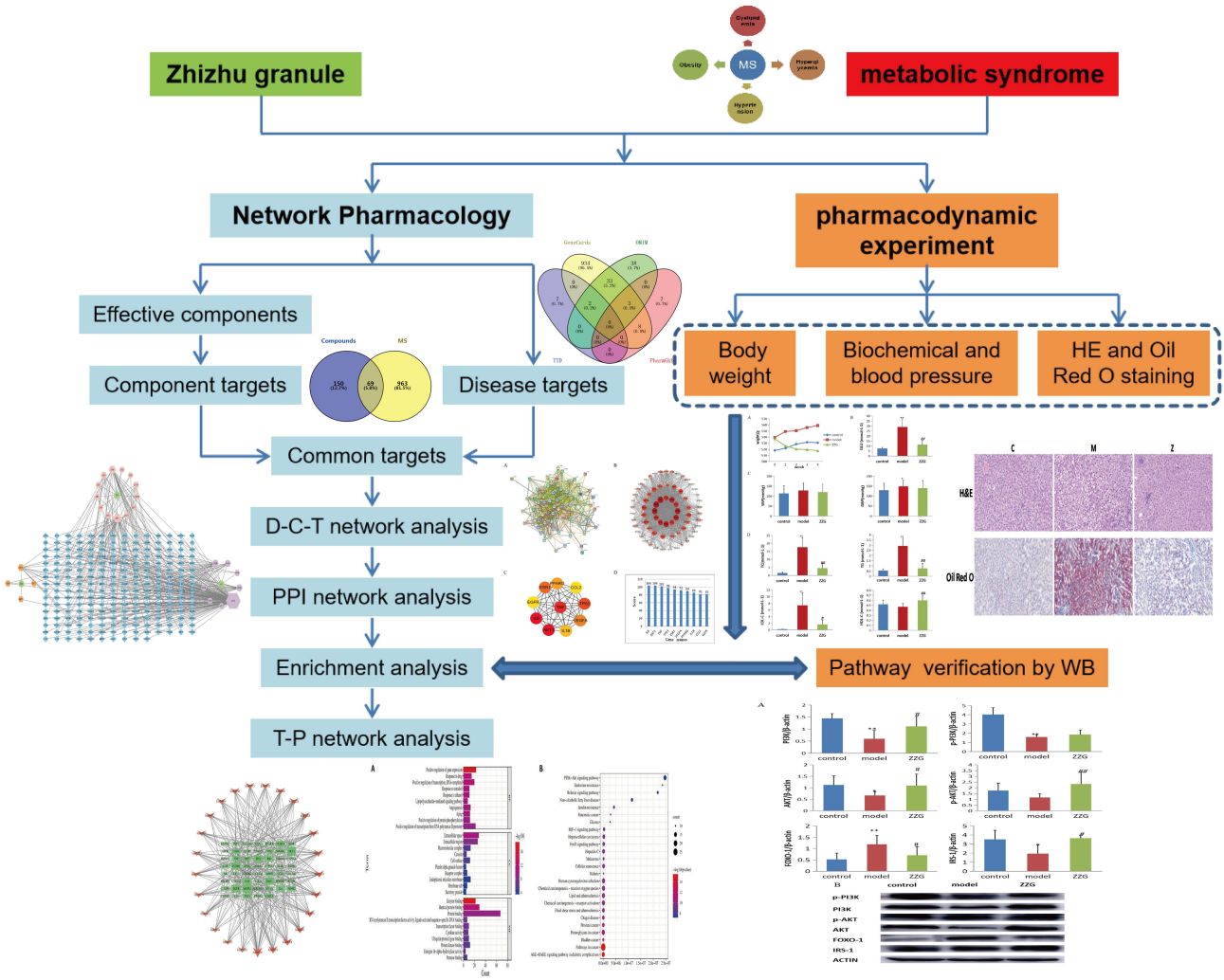

Figure 1 Flow chart showing the detailed process of this study. 1–1. The main clinical symptoms of MS. 1–2. Venn diagram of the number of target proteins in four databases. 1–3. Venn diagram for the common targets of drug and MS. 1–4. PPI network of ZZG against MS. 1–5. The drug-component-target network of ZZG against MS. 1–6. The target-pathway network of ZZG against MS. 1–7. The enrichment analysis of the 69 potential therapeutic targets. 1–8. Analysis of body weight, Biochemical and blood pressure of rats. 1–9. HE staining and oil red O staining of the rats' liver tissue. 1–10. Analysis of the protein expression levels on PI3K/AKT signaling pathway. See the corresponding figure in the manuscript for a detailed description.

drugs. The flow chart of the study is shown in Figure 1. All the steps and results of the network pharmacology and pharmacodynamic experiment are presented in this figure.

Materials and Methods

Network Pharmacology

Data Preparation

The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP; https://old.tcmsp-e.com/tcmsp.ph),[21] a unique Chinese herbal medicine system pharmacology platform, was used to obtain the compounds of Zhishi, Baizhu, and Heye. However, as not all compounds from medicinal materials exhibit drug characteristics, oral bioavailability (OB) and drug-likeness (DL) were used to screen candidate compounds obtained from the TCMSP to identify the compounds influencing MS. High OB values often constitute an important consideration for the development of bioactive molecules as therapeutic agents. DL evaluation is used in drug design to evaluate whether a compound is chemically suitable to be used as a drug and how drug-like molecule acts with respect to parameters affecting its pharmacodynamic and pharmacokinetic profiles, which ultimately impact its absorption, distribution, metabolism, and

excretion properties.[22] The molecules with OB ≥ 30% and DL ≥ 0.18 are considered to exhibit relatively better pharmacological properties. After the screening, the protein targets information of the effective chemical components was queried, and the target names were entered in the Uniprot database for standardization (https://www.uniprot.org/).[23]

"Metabolic syndrome" was used as a keyword to search and collect MS-related target genes in following four databases, containing genes and variants associated with human diseases: the GeneCards Database (https://www.genecards.org/),[24] the Online Mendelian Inheritance in Man database (OMIM, https://www.omim.org/),[25] the Therapeutic Target Database (TTD, http://bidd.nus.edu.sg/group/cjttd),[26] and the Pharmacogenetics and Pharmacogenomics Knowledge Base (PharmGKB, https://www.pharmgkb.org)[27]. All the above results were imported into EXCEL, and deduplicated after merging data, after which the ultimate disease targets were obtained. However, the searched targets overlapped with part of the MS target genes and the targets of ZZG. Venny 2.1.0 online software drawing tool platform (https://bioinfogp.cnb.csic.es/tools/venny) was used to analyze data and draw the Venn diagram, finally generating their intersection targets.

Protein-Protein Interaction(PPI) Network Analysis

To further study how ZZG targets interact with MS-related genes and identify the core regulatory targets, the overlapping targets of the compounds in ZZG and MS were submitted to the STRING database (https://cn.string-db.org/),[28] a functional protein association network with the largest number of protein data sources. In this system, we set the species to "Homo sapiens" and selected the confidence to ≥ 0.4, ran the software, and obtained the PPI results. Subsequently, we exported the data, imported them into Cytoscape 3.9.1 to construct and visualize the PPI network, and then analyzed pharmacological significance. The NetworkAnalyzer plugin in Cytoscape software was used to calculate topology parameters, after which the PPI network structure was adjusted according to the Degree to make it more intuitive. Finally, the CytoHubba plugin in Cytoscape software was used to search for the top 10 target proteins with Degree as core targets.

Pathway and Function Enrichment Analysis

To better understand the processes associated with MS and ZZG treatment, we performed the Gene Ontology (GO) function enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The overlapping targets of the compounds in ZZG and MS were entered into the Database for Annotation, Visualization, and Integrated Discovery (DAVID, https//david.ncifcrf.gov/)[29] database to find enriched genes, after which GO function enrichment and KEGG pathway enrichment results were downloaded. DAVID is a bioinformation database that provides systematic and integrated biological function annotation information for large-scale genes or proteins to help users extract biological information from them. Currently, the DAVID database is mainly used for functional and pathway enrichment analysis of differential genes. The GO enrichment analysis, consisting of three parts, namely biological process (BP), molecular function (MF), and cellular component (CC), was used to describe gene attributes. The KEGG is a database integrating genomic, chemical, and system functional information that can be used for large-scale systematic analysis of molecular interaction networks of genes or proteins. Weishengxin's online software drawing platform was used to draw enrichment bar charts and enrichment bubble charts to visualize the data.

Network Construction

In addition to constructing a PPI network, drug-compound-target (D-C-T) and target-pathway (T-P) networks were also constructed to further explore the correlation between drugs and diseases. D-C-T relationship was established with three herbs of ZZG and their effective components, as well as the overlapping targets of the compounds in ZZG and MS. T-P relationship was established with the overlapping targets and the top 25 pathways from KEGG enrichment. Finally, the graphical and diagrammatic visualized networks were constructed using Cytoscape software.

Pharmacodynamic Experiment

Preparations of Animals and Drugs

Healthy male Sprague-Dawley rats (n=34, weighing 200±10g, 6 weeks old) were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd (SCXK 2019–0009). They were kept under specific pathogen-free conditions at

constant temperature and humidity. The Welfare and Ethical Committee for Research Involving Animals of Experimental Research Center, China Academy of Chinese Medicine Science gave approval for the research (ERCCAMS21-2109-02). ZZG was purchased from Nanjing Zhongshan Pharmaceutical Co., LTD (batch number: 210902), and it conformed to the quality standards of the 2020 edition of the "Pharmacopeia of the People's Republic of China". Per 1000 g ZZG contains Zhishi 333g, Baizhu 666g, Heye 100g. All of them were decocted 2 hours twice with water and added 70% ethanol after concentrating. Finally, it would be condensed into a paste with a relative density of 1.30~1.35 (60°C). Each bag of ZZG contains 83mg *Naringin* ($C_{19}H_{21}NO_2$) and 75mg *Neohesperidin* ($C_{28}H_{21}O_{15}$). The obtained Thin layer chromatography (TLC) spots of Zhishi, Baizhu and Heye in ZZG occurred at the corresponding positions compared to the controls.

Reagents and Materials

Streptozotocin (STZ) was purchased from Bioruler (Connecticut, USA). 0.1M Sodium citrate-Hydrochloric acid buffer was provided by Solarbio (Beijing, China). Bicinchoninic acid (BCA) protein assay kit, PMSF and RIPA lysis buffer were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Antibodies against AKT, p-AKT, PI3K, IRS-1, FOXO1 and beta-actin were provided by Proteintech Group, Inc. (Chicago, USA). Antibodies against p-PI3K were obtained from Abcam (Cambridge, UK).

Establishment of MS Rat Model and Drug Administration

After adaptive feeding for one week, the rats were randomly divided into the control group (n=10) and the model group (n=24). The control group was fed with basic diet, and the model group was fed with high-sugar-fat-salt diet (HSFSD, 50% basal feed, 10% egg yolk powder, 2% cholesterol, 7.5% milk powder, 10% fructose, 5% palmitic acid, 3% salt, 2% fish meal, 0.5% bile salts).[10] After 12 weeks of intervention, the model group was intraperitoneally injected with STZ solution, making up STZ and 0.1M sodium citrate-hydrochloric acid buffer, at a dose of 25 mg/kg, while the control group was injected with the same volume of saline, once a day for three consecutive days. Next, according to the clinical characteristics of MS, the abdomen circumference (AC), glucose (GLU), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), systolic blood pressure (SBP), and diastolic blood pressure (DBP) levels of model group higher than the value of mean plus standard deviation (SD) of the control group were selected as the MS model rats. The twenty successful MS model rats were randomly divided into the ZZG group (n=10) and the model group (n=10). The control group was fed with a basic diet and distilled water, and the model group was fed with HSFSD and distilled water, while the ZZG group received HSFSD and ZZG (3.24g/kg/day) treatment for 4 weeks.

Biochemical and Blood Pressure Examination

Before treatment and euthanasia, blood pressure in rats was measured respectively through uninjured arteria caudalis. After treatment, the blood and liver of each rat were collected. Blood was centrifuged in a cryogenic centrifuge at 3,500 rmp for 15 min, after which the total cholesterol (TC), TG, HDL-C, and low-density lipoprotein cholesterol (LDL-C) were determined. The liver was used for subsequent experiments.

Histological Examination

The liver tissue of rats was fixed in 4% paraformaldehyde sectioned, and the tissue sections were subjected to HE staining and Oil Red O staining. We selected three sections from each group to examine and measure under a light microscope, after which we compared the morphology of liver tissue of rats in each group and calculated lipid volume fraction (lipid area/total area×100%).

Western Blot

First, 100g liver tissue was weighed and homogenized, after which the total protein was extracted by adding RIPA buffer containing PMSF. Second, the protein concentration was tested by the BCA protein assay, after which the sample volume was calculated and determined. Third, proteins were subjected to SDS-PAGE gel electrophoresis and transferred to PVDF membranes. The membranes were blocked in 5% skim milk for 2h, after which they were incubated overnight at 4°C with the primary antibodies. The antibodies included beta-actin (1:1000), AKT (1:3000), p-AKT (1:3000), PI3K (1:5000), p-PI3K (1:1000), FOXO1 (1:1000), IRS-1 (1:1000). Then, membranes were washed 3 times with 1×TBST buffer for 10 minutes each time, and incubated with HRP-conjugated secondary antibody (1:3000) at room temperature

for 2h. Finally, the proteins were visualized using ECL system, and Gray-scale levels of the protein bands were analyzed using Image J.

Statistical Analysis

The results of all validation experiments were analyzed by software SPSS20.0. First, the normal distribution of all variables were tested by SPSS. For variables whose distribution differs from the normal distribution, the median and interquartile range were used. For variables whose distribution does not differ significantly from the normal distribution, the mean and standard deviation were used. Then, for comparison among multiple groups of samples, one-way analysis of variance (ANOVA) was used. If the variance was homogeneous, LSD method was used for test; if the variance was not homogeneous, Dunnett's method was used for analysis. A value of $p<0.05$ indicated statistical significance, and $p<0.01$ was considered highly significant.

Results

Network Pharmacology results

Active Compounds and Targets of ZZG

Highly active components were screened according to the pharmacokinetic parameters of drug absorption, distribution, metabolism, and excretion in the human body. The screening criteria were OB ≥ 30%, DL ≥ 0.18, and the components without corresponding targets were excluded. Finally, 22 kinds of active compounds of Zhishi, 7 kinds of active compounds of Baizhu, and 15 kinds of active compounds of Heye were obtained. There were 125 targets of active components of Zhishi, 19 targets of Baizhu, and 210 targets of Heye. After removing the duplicate targets, and those that could not be found in the Uniprot database, a total of 219 targets were obtained.

Targets of MS and Overlapping Targets

"Metabolic syndrome" was entered into the disease database to search for disease targets. Our results showed 10 targets in TTD, 76 targets in OMIM, 18 targets in PharmGKB, and 15677 targets in the GeneCards database. Also, 980 targets were obtained by screening four times according to the median. The combination of the 4 databases was taken, and after the duplications were deleted, 1032 disease targets were obtained (Figure 2A). The online software Venny 2.1.0 was used to input 219 drug and 1032 disease targets, generating 69 drug-disease common targets after intersecting (Figure 2B).

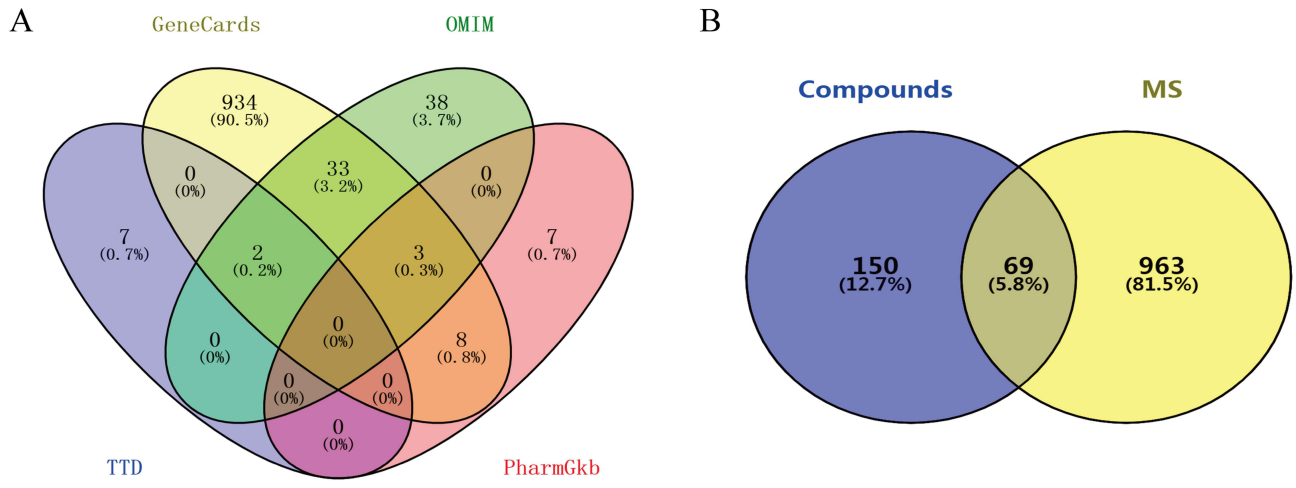

Figure 2 Target proteins of ZZG and drug-disease common targets. **(A)** Venn diagram of the number of target proteins in four databases. The blue ovals represent disease targets obtained from the TTD database. The yellow ovals represent disease targets obtained from the GeneCards database; the green ovals represent disease targets obtained from the OMIM database; pink ovals represent disease targets obtained from the PharmGKB database; overlapping areas represent the overlapping targets of the four databases; unoverlapped areas represent targets that are individually owned by each database. **(B)** Venn diagram for the common targets of drug and MS. The blue circle represents the targets of the drug compounds; the yellow circle represents the targets of MS. The overlapping area represents the common targets of drugs and MS.

PPI Network Analysis

After submitting the 69 common target proteins into the STRING database, the confidence was set to 0.4 for analysis. Next, we obtained the protein interaction relationship data containing 69 nodes and 1698 edges (Figure 3A). They were imported into Cytoscape software for analyzing and constructing the PPI network, and the targets were adjusted according to the Degree value (Figure 3B). After analyzing the PPI network by NetworkAnalyzer, the top 10 hub genes with CytoHubba were screened by degree values: IL6, AKT1, TNF, TP53, ESR1, VEGFA, PPARG, IL1β, CCL2, and EGFR (Figure 3C and D). As core targets, these proteins may be the key targets of ZZG against MS.

D-C-T Network Analysis

The active components and 69 drug targets in ZZG were input into Cytoscape software, and the "drug-component-target" network diagram was drawn. Drug-active ingredients were ranked by degree. The top three key chemical ingredients were quercetin (MOL000098, degree=139), luteolin (MOL000006, degree=36), and naringenin (MOL004328, degree=27) (Figure 4A).

T-P Network Analysis

To further elucidate the molecular mechanism of ZZG against MS, T-P network was established based on the top 25 signaling pathways and the involved targets. Cytoscape software was used to draw the obtained pathway and target information into a network diagram, and the degree was set according to the number of connected nodes. The core targets in the network were VEGFA, AKT1, TNF, MET, TP53, RB1, CCND1, EGF, RAF1, EGFR, MAPK1, IL6, MDM2, PTEN, CXCL8, etc. Among these target genes, except TNF, RB1, and CXCL8, the others were in PI3K/AKT signaling pathway (Figure 4B).

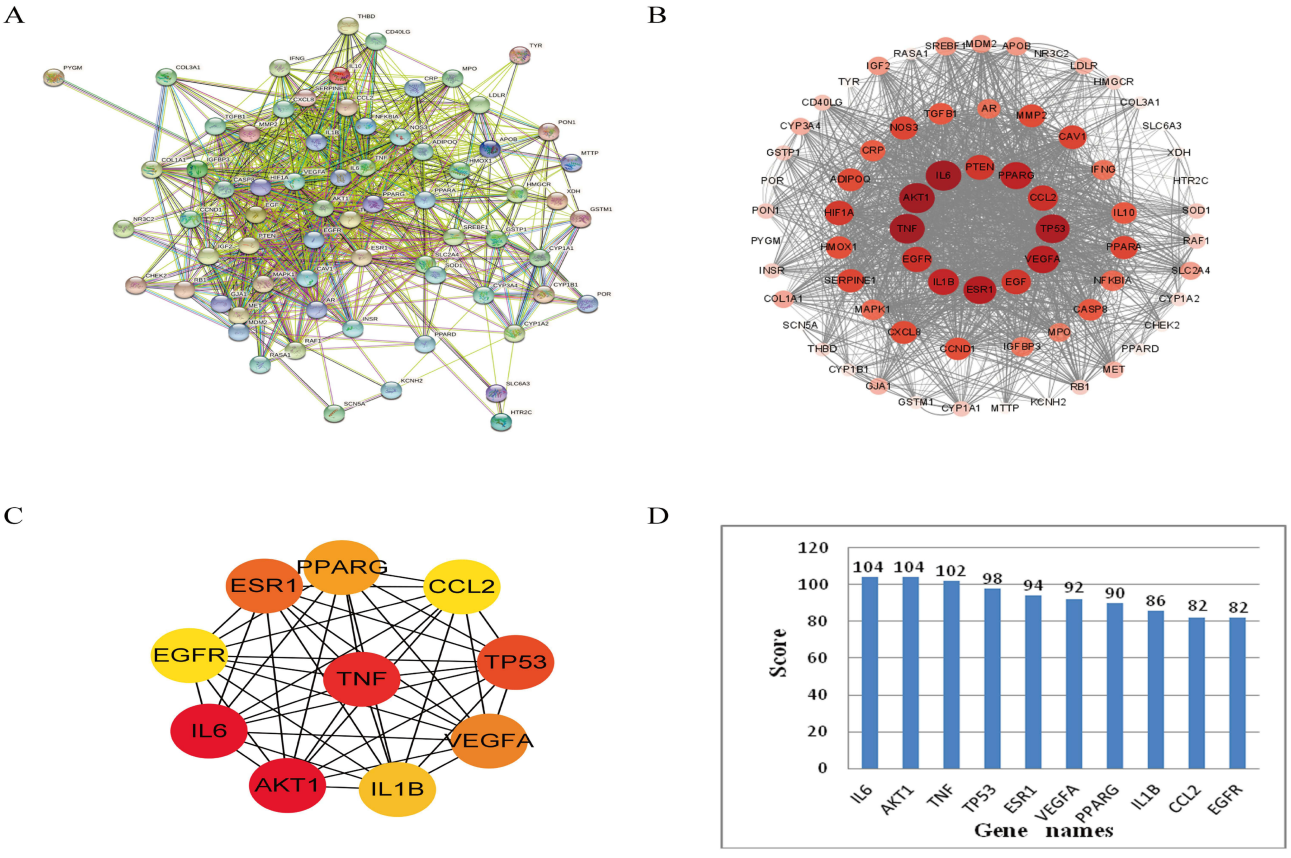

Figure 3 PPI network of ZZG against MS. **(A)** PPI network of protein targets obtained from the STRING database. **(B)** PPI network constructed by Cytoscape. The size of the nodes is proportional to the values of the degree. As the nodes' color becomes lighter, the degree values become gradually smaller. **(C)** The top 10 hub genes cluster. **(D)** Sequencing of hub genes by degree values.

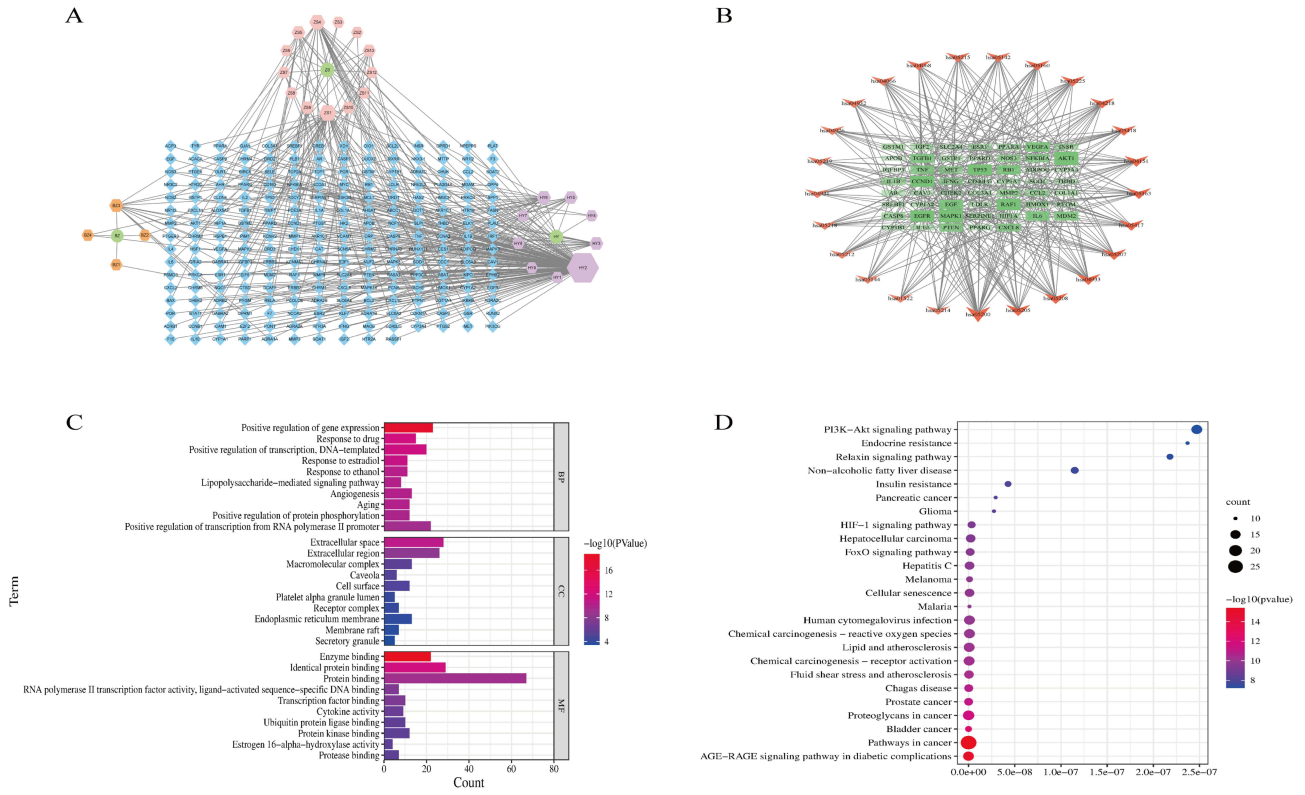

Figure 4 Analysis of key ingredients and signaling pathways of ZZG against MS. **(A)**The drug-component-target network of ZZG against MS. The green circle represents the three drugs of ZZG, with ZS representing Zhishi, BZ representing Baizhu, and HY representing Heye. The hexagon represents the active components of the three drugs, and the blue diamonds represent the target genes of ZZG against MS. The number of edges connected to the node represents the degree of freedom. **(B)**The target-pathway network of ZZG against MS. The red nodes represent the signaling pathways, and the green node represents the involved targets. The larger the shape and the more prominent the color of the targets, the more significant the enrichment. **(C)** The GO enrichment analysis of the 69 potential therapeutic targets. **(D)** The KEGG pathway enrichment analysis of the 69 potential therapeutic targets. The circle size represents the number of genes enriched in each KEGG pathway term, and the different colors represent the *P*-value.

Enrichment Analysis

To identify the relevant biological functions of ZZG against MS, GO enrichment analysis of the 69 potential therapeutic targets was performed. The top 10 significantly enriched terms with a greater number of involved targets in each part of GO enrichment are shown, indicating that ZZG may exert its therapeutic effects against MS through positive regulation of gene expression, drug response, extracellular space, extracellular region, enzyme binding and protein binding (Figure 4C). KEGG pathway enrichment analysis of the 69 potential therapeutic targets was performed to explore the potential pathways of ZZG against MS. According to the order of P value, the top 25 significantly enriched pathways were selected. Among these, we focused on the PI3K/AKT signaling pathway, which was the most potential pathway that ZZG used against MS (Figure 4D).

Experimental Validation

Body Weight Results

Body weight can reflect the degree of obesity in rats. Compared with the control group, the body weight of rats in the model group increased ($p<0.01$ or $p<0.05$). Compared with the model group, before the intervention, the body weight of rats in the ZZG group was not different; however, after the intervention, it significantly decreased ($p<0.01$) (Figure 5A).

Biochemical and Blood Pressure Results

Metabolic indicators such as blood glucose, blood lipid, and blood pressure can reflect the metabolic state of the body. Results showed that TC, TG, LDL-C, GLU, and DBP were significantly increased in the model group compared with the

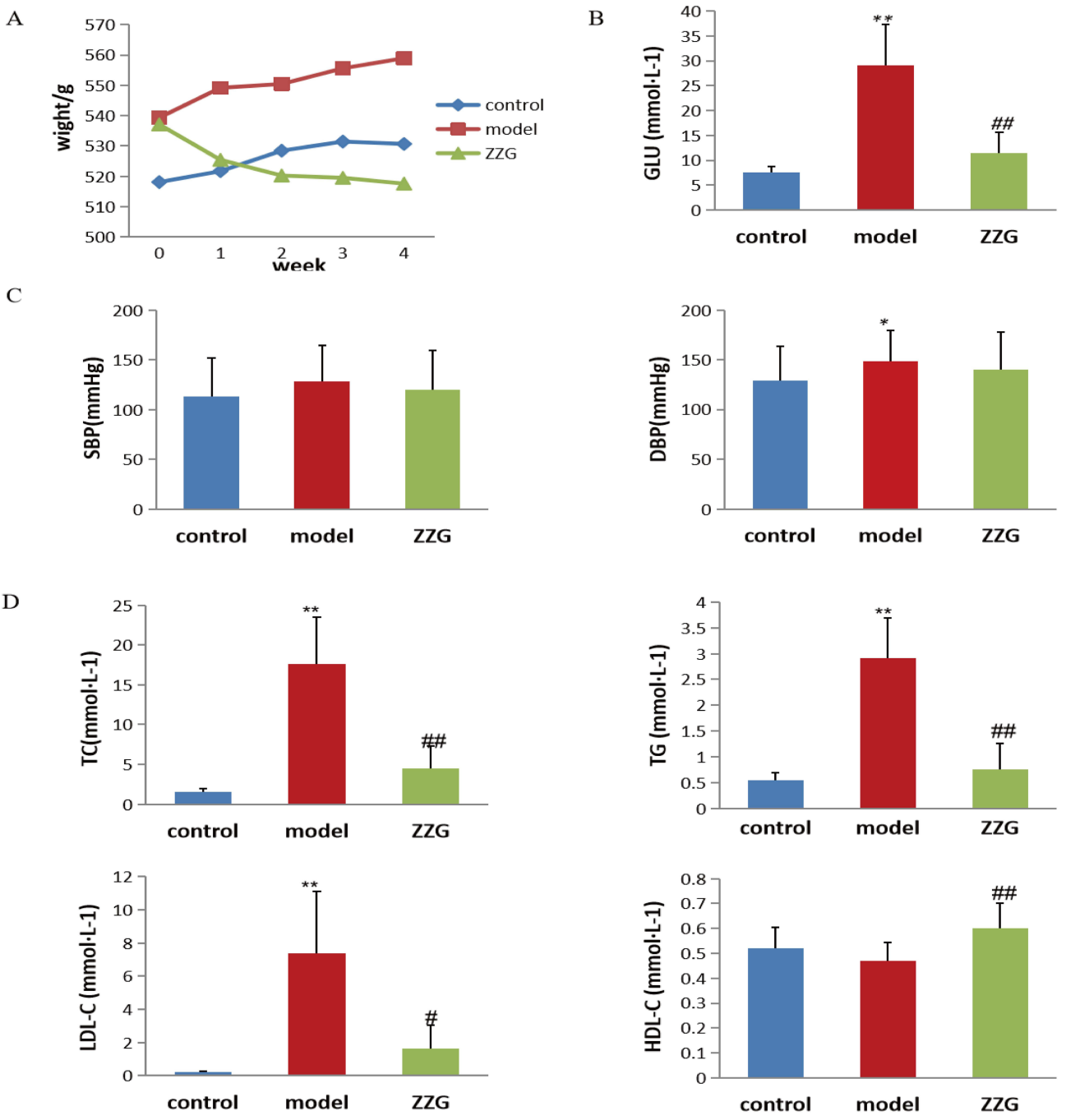

Figure 5 Analysis of body weight, Biochemical and blood pressure of rats. (**A**) The figure of body weight change in rats. (**B**) Analysis of blood glucose in rats. (**C**) Analysis of blood pressure in rats. (**D**) Analysis of blood lipid in rats. *$P < 0.05$ vs control group, **$P < 0.01$ vs control group, $^{\#}P < 0.05$ vs model group, $^{\#\#}P < 0.01$ vs model group.

normal control group ($p<0.01$ or $p<0.05$). Compared with the model group, TC, TG, LDL-C, and GLU in the ZZG group were significantly decreased (all $p<0.01$) (Figure 5B–D).

HE and Oil Red O Staining

Compared with the normal control group, a large number of vacuoles of different sizes appeared in the hepatocytes of the model group. Also, some cell nuclei were even squeezed to the edge of the cells by the vacuoles. In addition, Oil Red O staining showed that the reddened area of liver tissue increased, suggesting the increase of hepatocyte lipid droplets

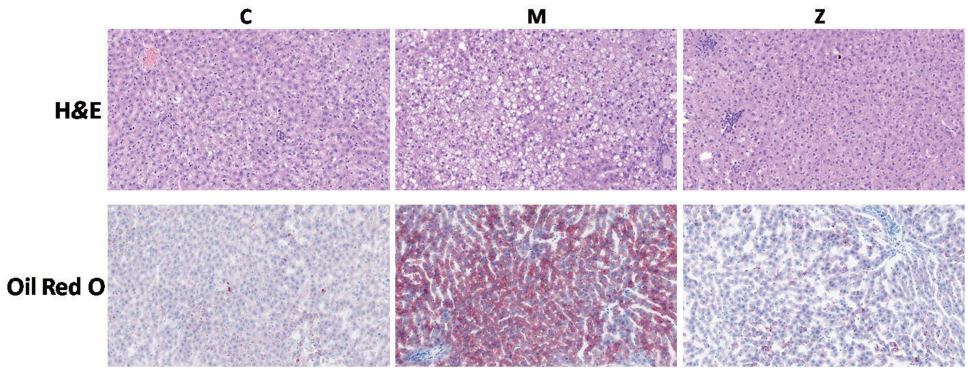

Figure 6 HE staining and oil red O staining of the rats' liver tissue (200×). In H&E staining, the vacuoles indicate lipid droplets in the hepatocyte, and the more vacuoles, the more serious the hepatocyte steatosis. In Oil Red O staining, the more reddened area indicates more lipid droplets. (**C**) control group, (**M**) model group, (**Z**) ZZG group.

and hepatocyte steatosis. Compared with the model group, alleviated hepatocyte steatosis and decreased reddened area of liver tissue and liver lipid droplet deposition were observed in the ZZG group (Figure 6).

Western Blot

Western blot analysis indicated that compared with the control group, the expression levels of PI3K, AKT, and IRS-1 protein in liver tissue of the MS model group were significantly decreased, whereas those of FOXO-1 were significantly increased ($p<0.01$ or $p<0.05$). ZZG restored the MS-induced protein expression levels of PI3K, AKT, and IRS-1 and simultaneously abrogated FOXO-1 expression ($p<0.05$). Compared with the control group, the expression level of p-PI3K protein in the model group decreased ($p<0.01$). Although ZZG increased it, no statistical significance was found. On the contrary, the expression levels of p-AKT were not significantly decreased in the model group but were significantly restored by ZZG ($p<0.01$) (Figure 7).

Discussion

MS is a non-communicable disease affecting a great number of individuals worldwide and becoming an important cause of morbidity and mortality. MS has pathologic characteristics such as abdominal obesity, insulin resistance, hypertension, and hyperlipidemia. According to the definition of MS formulated by IDF in 2005, waist, GLU, TG, and blood pressure levels are elevated, and HDL-C levels are decreased in patients with MS.[1] In this study, the therapeutic effect of ZZG on MS was tested using animal experiments, and our results showed that ZZG could significantly decrease the body weight, TC, TG, LDL-C, and GLU levels in MS rats. The liver is the main organ of endogenous lipid synthesis that has an important role in the regulation of lipid homeostasis.[30] The liver synthesizes fat from fatty acids taken up in plasma and secretes fat into plasma through very low-density lipoproteins to regulate blood lipids.[31] It was found that ZZG could alleviate hepatocyte steatosis and decrease liver lipid droplet deposition. Thus, ZZG could affect the treatment of MS to a certain extent by improving glucose-lipid metabolism.

Network pharmacology methods clearly and systematically revealed the complex interactions between active ingredients of drugs and targets of diseases and the potential therapeutic mechanisms for ZZG treating MS. Our results showed that 10 hub genes were closely related to the pathogenesis of MS, most of which are known to cause the glucose and lipid metabolism disorders in the body. Low-grade inflammation is a significant factor in MS. A previous study reported increased levels of IL-6 and TNF-αin patients with MS.[32] The inflammatory response can cause insulin resistance and obesity, likely contributing to MS occurrence.[33,34] The inhibition of IL-1β-mediated action can promote pancreatic beta-cell function, thus improving insulin resistance.[35] CCl2 has a vital role in the process of inflammation, and elevated levels of CCL2 increase the risk of hypertension, hyperlipidemia, type 2 diabetes, and coronary heart disease.[36] AKT1 is a serine/threonine-specific protein kinase, which can be stimulated by insulin and insulin-like growth factor 1(IGF1). It can also affect glucose metabolism.[37] PPARG has been reported to participate in lipid metabolism regulation, ie, the de novo synthesis of fatty acids, phospholipids, and sphingolipids.[38] The other 4 core genes, TP53,

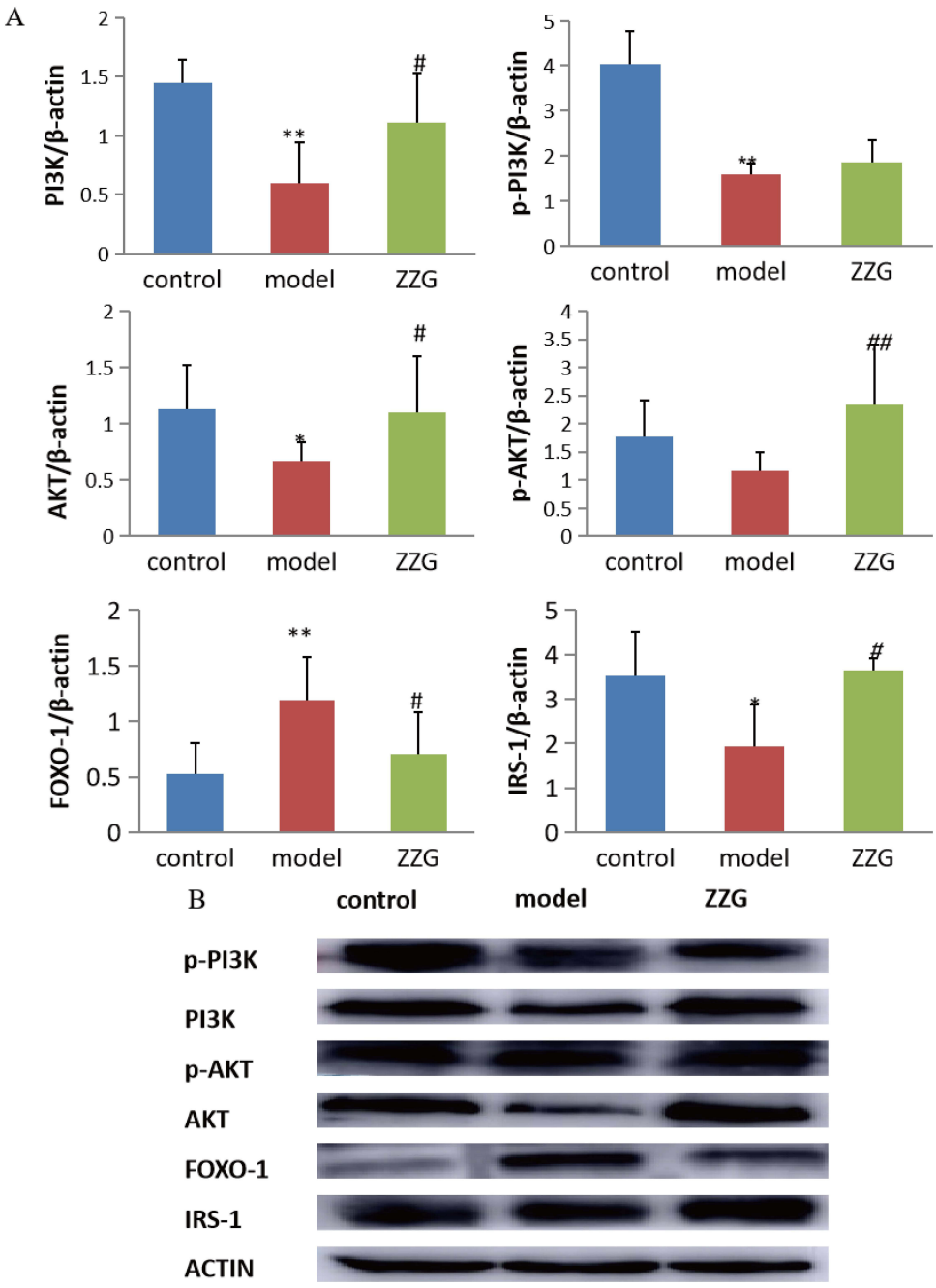

Figure 7 Analysis of the protein expression levels on PI3K/AKT signaling pathway. **(A)** Bar graph shows the relative protein expression level of p-PI3K, PI3K, p-AKT, AKT, FOXO-1, and IRS-1 in the liver tissue of rats. $^{*}P < 0.05$ vs control group, $^{**}P < 0.01$ vs control group, $^{\#}P < 0.05$ vs model group, $^{\#\#}P < 0.01$ vs model group. **(B)** The expression of the above proteins. (**C**) control group, (**M**) model group, (**Z**) ZZG group.

ESR1, VEGFA, and EGFR, could be involved in the pathological mechanism of MS directly or indirectly; however, their role in MS and the effect of ZZG should be further investigated.

PI3K/AKT signaling pathway resulted as the most potential pathway that ZZG uses against MS in our KEGG pathway analysis. The PI3K/AKT signaling pathway is an intracellular signaling pathway important in the cell cycle process. It has been associated with cell proliferation, apoptosis, metabolism, and cancer.[39] This signaling pathway regulates glucose metabolism by participating in the reduction of glycogen synthesis. It also strengthens glycolysis and regulates lipogenesis by inhibiting sterol regulatory element binding transcription factor (SREBP-1c).[40,41] Our findings showed that ZZG could promote glucose-lipid metabolism in rats with MS through upregulating PI3K/AKT pathway

since ZZG significantly increased PI3K, AKT, and p-AKT protein expression. Although there was no statistical significance, the p-PI3K protein expression level was slightly increased, suggesting that ZZG probably upregulates p-PI3K.

Furthermore, the mechanism of ZZG in treating MS based on network pharmacology analysis was verified by animal experiments. Studies have shown that FOXOs are a crucial downstream factor of the PI3K/AKT signaling pathway.[42] FOXO-1 can limit mitochondrial glucose oxidation and increase lipid uptake, while its expression/activity can be inhibited by insulin through the PI3K/AKT signaling pathway.[43] Our results showed that ZZG could abrogate the protein expression of FOXO-1 induced by MS. IRS-1 acts as the principal insulin signaling protein and crucial upstream factor of the PI3K/AKT signaling pathway. The decrease of IRS-1 can alleviate the impaired insulin signaling pathway through PI3K/AKT signaling pathway.[44,45] In the present study, we found that ZZG restored the MS-induced protein expression of IRS-1, which is consistent with the results of previous studies.

Conclusion

Comprehensive, systematic research into network pharmacology is consistent with the perspective of holisticity, which is a main characteristic of TCM. Compared to single compounds, TCM prescription, which frequently contains various herbs or other components, has a synergistic effect in effecting a cure or reducing toxicity by acting at different levels on multiple targets and pathways. The results of network pharmacology elucidated the potential mechanisms of multitarget and multi-pathway of ZZG against MS. Meanwhile, the results of pharmacological experiments indicated that ZZG can improve glucose-lipid metabolism disorder in MS rats by ameliorating the relevant biochemical indicators and regulating the signaling pathways related to glucose-lipid metabolism. In conclusion, ZZG is a promising drug used to treat MS by promoting glucose-lipid metabolism. This study provides basic research support for the clinical application of ZZG against MS. Meanwhile, the study promoted the safety, efficacy, and mechanism investigations of ZZG, which then reinforces the credibility and popularity of it.

While the study provides significant insights into the effects of ZZG on metabolic syndrome in rats, the translation of these findings to human populations should be approached with caution. Besides, although we found the association of target genes and target proteins with ZZG against MS, but we have not explored the specific interactions between ZZG's active components and these molecular targets with molecular docking strategies or experimental research methods.In order to further elucidate the mechanism of ZZG against MS, in vitro experiments should be conducted. Also, future clinical studies should further validate the role of ZZG in MS, and observe potential long-term effects, safety, and tolerability of ZZG.

Data Sharing Statement

The data used to support the findings of the study are available on request from the authors.

Funding

This study was supported by the Research and Development fund projects of Experimental Research Center of China Academy of Chinese Medical Sciences [No.FZ2022001] and the National Natural Science Foundation of China [No.82074260] and the Fundamental Research Funds for the Central Public Welfare Research Institutes[No. XTCX2023003] and JBGS[2023006].

Disclosure

The authors declare that the study has no conflicts of interest.

References

1. Huang PL. A comprehensive definition for metabolic syndrome. *Dis Model Mech*. 2009;2(5–6):231–237. doi:10.1242/dmm.001180
2. Fahed G, Aoun L, Bou Zerdan M, et al. Metabolic Syndrome: updates on Pathophysiology and Management in 2021. *Int J Mol Sci*. 2022;23(2):786. doi:10.3390/ijms23020786
3. Saklayen MG. The Global Epidemic of the Metabolic Syndrome. *Curr Hypertens Rep*. 2018;20(2):12. doi:10.1007/s11906-018-0812-z

4. Hirode G, Wong RJ. Trends in the Prevalence of Metabolic Syndrome in the United States, 2011-2016. *JAMA*. 2020;323(24):2526–2528. doi:10.1001/jama.2020.4501
5. Samson SL, Garber AJ. Metabolic syndrome. *Endocrinol Metab Clin North Am*. 2014;43(1):1–23. doi:10.1016/j.ecl.2013.09.009
6. Yanai H, Adachi H, Hakoshima M, Katsuyama H. Molecular Biological and Clinical Understanding of the Pathophysiology and Treatments of Hyperuricemia and Its Association with Metabolic Syndrome, Cardiovascular Diseases and Chronic Kidney Disease. *Int J Mol Sci*. 2021;22(17):9221. doi:10.3390/ijms22179221
7. Kalra S, Aggarwal S, Khandelwal D. Thyroid Dysfunction and Dysmetabolic Syndrome: the Need for Enhanced Thyrovigilance Strategies. *Int J Endocrinol*. 2021;9641846. doi:10.1155/2021/9641846
8. Yki-Järvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. *Lancet Diabetes Endocrinol*. 2014;2(11):901–910. doi:10.1016/S2213-8587(14)70032-4
9. Lotti F, Marchiani S, Corona G, Maggi M. Metabolic Syndrome and Reproduction. *Int J Mol Sci*. 2021;22(4):1988. doi:10.3390/ijms22041988
10. Chen X, Yan XR, Liu J, Zhang LP. Chaiqi decoction ameliorates vascular endothelial injury in metabolic syndrome by upregulating autophagy. *Am J Transl Res*. 2020;12(9):4902–4922.
11. Wu H, Jing Z, Tang X, et al. To compare the efficacy of two kinds of Zhizhu pills in the treatment of functional dyspepsia of spleen-deficiency and qi-stagnation syndrome: a randomized group sequential comparative trial. *BMC Gastroenterol*. 2011;11:81. doi:10.1186/1471-230X-11-81
12. Shu Y, He D, Li W, et al. Hepatoprotective Effect of Citrus aurantium L. Against APAP-induced Liver Injury by Regulating Liver Lipid Metabolism and Apoptosis. *Int J Biol Sci*. 2020;16(5):752–765. doi:10.7150/ijbs.40612
13. Jiang YH, Zhang P, Tao Y, et al. Banxia Baizhu Tianma decoction attenuates obesity-related hypertension. *J Ethnopharmacol*. 2021;266:113453. doi:10.1016/j.jep.2020.113453
14. Yang L, Yu H, Hou A, et al. A Review of the Ethnopharmacology, Phytochemistry, Pharmacology, Application, Quality Control, Processing, Toxicology, and Pharmacokinetics of the Dried Rhizome of Atractylodes macrocephala. *Front Pharmacol*. 2021;12:727154. doi:10.3389/fphar.2021.727154
15. Zhao S, Liu Z, Wang M, et al. Anti-inflammatory effects of Zhishi and Zhiqiao revealed by network pharmacology integrated with molecular mechanism and metabolomics studies. *Phytomedicine*. 2018;50:61–72. doi:10.1016/j.phymed.2018.09.184
16. Xu N, Li M, Wang P, Wang S, Shi H. Spectrum-Effect Relationship Between Antioxidant and Anti-inflammatory Effects of Banxia Baizhu Tianma Decoction: an Identification Method of Active Substances With Endothelial Cell Protective Effect. *Front Pharmacol*. 2022;13:823341. doi:10.3389/fphar.2022.823341
17. Zhang C, Deng J, Liu D, et al. Nuciferine ameliorates hepatic steatosis in high-fat diet/streptozocin-induced diabetic mice through a PPARα/PPARγ coactivator-1α pathway. *Br J Pharmacol*. 2018;175(22):4218–4228. doi:10.1111/bph.14482
18. Dong Y, Zhao Q, Wang Y. Network pharmacology-based investigation of potential targets of astragalus membranaceous-angelica sinensis compound acting on diabetic nephropathy. *Sci Rep*. 2021;11(1):19496. doi:10.1038/s41598-021-98925-6
19. Yuan H, Ma Q, Cui H, et al. How Can Synergism of Traditional Medicines Benefit from Network Pharmacology? *Molecules*. 2017;22(7):1135. doi:10.3390/molecules22071135
20. Li X, Liu Z, Liao J, Chen Q, Lu X, Fan X. Network pharmacology approaches for research of Traditional Chinese Medicines. *Chin J Nat Med*. 2023;21(5):323–332. doi:10.1016/S1875-5364(23)60429-7
21. Ru J, Li P, Wang J, et al. TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. *J Cheminform*. 2014;6:13. doi:10.1186/1758-2946-6-13
22. Zheng W, Wu J, Gu J, et al. Modular Characteristics and Mechanism of Action of Herbs for Endometriosis Treatment in Chinese Medicine: a Data Mining and Network Pharmacology-Based Identification. *Front Pharmacol*. 2020;11:147. doi:10.3389/fphar.2020.00147
23. Zhu N, Hou J, Ma G, Liu J. Network Pharmacology Identifies the Mechanisms of Action of Shaoyao Gancao Decoction in the Treatment of Osteoarthritis. *Med Sci Monit*. 2019;25:6051–6073. doi:10.12659/MSM.915821
24. Safran M, Rosen N, Twik M, et al. The GeneCards Suite Chapter. *Practical Guid Life Scie Datab*. 2022;27–56.
25. Rasmussen SA, Hamosh A, Curators OMIM. What's in a name? Issues to consider when naming Mendelian disorders. *Genet Med*. 2020;22(10):1573–1575. doi:10.1038/s41436-020-0851-0
26. Tao L, Wang B, Zhong Y, et al. Database and Bioinformatics Studies of Probiotics. *J Agric Food Chem*. 2017;65(35):7599–7606. doi:10.1021/acs.jafc.7b01815
27. Maillard M, Gong L, Nishii R, Yang JJ, Whirl-Carrillo M, Klein TE. PharmGKB summary: acyclovir/ganciclovir pathway. *Pharmacogenet Genomics*. 2022;32(5):201–208. doi:10.1097/FPC.0000000000000474
28. Szklarczyk D, Gable AL, Nastou KC, et al. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res*. 2021;49(D1):D605–D612. doi:10.1093/nar/gkaa1074
29. Sherman BT, Hao M, Qiu J, et al. DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acids Res*. 2022;50(W1):W216–21. doi:10.1093/nar/gkac194
30. Borén J, Taskinen MR, Björnson E, Packard CJ. Metabolism of triglyceride-rich lipoproteins in health and dyslipidaemia. *Nat Rev Cardiol*. 2022;19(9):577–592. doi:10.1038/s41569-022-00676-y
31. Alves-Bezerra M, Cohen DE. Triglyceride Metabolism in the Liver. *Compr Physiol*. 2017;8(1):1–8. doi:10.1002/cphy.c170012
32. Cao C, Su M. Effects of berberine on glucose-lipid metabolism, inflammatory factors and insulin resistance in patients with metabolic syndrome. *Exp Ther Med*. 2019;17(4):3009–3014.
33. Lontchi-Yimagou E, Sobngwi E, Matsha TE, Kengne AP. Diabetes mellitus and inflammation. *Curr Diab Rep*. 2013;13(3):435–444. doi:10.1007/s11892-013-0375-y
34. Villarroya F, Cereijo R, Gavaldà-Navarro A, Villarroya J, Giralt M. Inflammation of brown/beige adipose tissues in obesity and metabolic disease. *J Intern Med*. 2018;284(5):492–504. doi:10.1111/joim.12803
35. Luotola K. IL-1 Receptor Antagonist (IL-1Ra) Levels and Management of Metabolic Disorders. *Nutrients*. 2022;14(16):3422. doi:10.3390/nu14163422
36. Singh S, Anshita D, Ravichandiran V. MCP-1: function, regulation, and involvement in disease. *Int Immunopharmacol*. 2021;101(Pt B):107598. doi:10.1016/j.intimp.2021.107598

37. George B, Gui B, Raguraman R, et al. AKT1 Transcriptomic Landscape in Breast Cancer Cells. *Cells*. 2022;11(15):2290. doi:10.3390/cells11152290
38. Ma S, Zhou B, Yang Q, et al. A Transcriptional Regulatory Loop of Master Regulator Transcription Factors, PPARG, and Fatty Acid Synthesis Promotes Esophageal Adenocarcinoma. *Cancer Res*. 2021;81(5):1216–1229. doi:10.1158/0008-5472.CAN-20-0652
39. Alzahrani AS. PI3K/AKT/mTOR inhibitors in cancer: at the bench and bedside. *Semin Cancer Biol*. 2019;59:125–132. doi:10.1016/j.semcancer.2019.07.009
40. Xie Y, Shi X, Sheng K, et al. PI3K/Akt signaling transduction pathway, erythropoiesis and glycolysis in hypoxia (Review). *Mol Med Rep*. 2019;19(2):783–791. doi:10.3892/mmr.2018.9713
41. Xiao H, Sun X, Lin Z, et al. Gentiopicroside targets PAQR3 to activate the PI3K/AKT signaling pathway and ameliorate disordered glucose and lipid metabolism. *Acta Pharm Sin B*. 2022;12(6):2887–2904. doi:10.1016/j.apsb.2021.12.023
42. Shi YY, Meng XT, Xu YN, Tian XJ. Role of FOXO protein's abnormal activation through PI3K/AKT pathway in platinum resistance of ovarian cancer. *J Obstet Gynaecol Res*. 2021;47(6):1946–1957. doi:10.1111/jog.14753
43. Chistiakov DA, Orekhov AN, Bobryshev YV. The impact of FOXO-1 to cardiac pathology in diabetes mellitus and diabetes-related metabolic abnormalities. *Int J Cardiol*. 2017;245:236–244. doi:10.1016/j.ijcard.2017.07.096
44. Bhattamisra SK, Koh HM, Lim SY, Choudhury H, Pandey M. Molecular and Biochemical Pathways of Catalpol in Alleviating Diabetes Mellitus and Its Complications. *Biomolecules*. 2021;11(2):323. doi:10.3390/biom11020323
45. Deng N, Guo R, Zheng B, Li T, Liu RH. IRS-1/PI3K/Akt pathway and miRNAs are involved in whole grain highland barley (Hordeum vulgare L.) ameliorating hyperglycemia of db/db mice. *Food Funct*. 2020;11(11):9535–9546. doi:10.1039/D0FO01990A