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Basic Study

Nardostachyos Radix et Rhizoma-Rhubarb in the treatment of diabetic kidney disease based on network pharmacology and experimental verification

Meng-Ying Che, Ling Yuan, Jiao Min, Duo-Jie Xu, Dou-Dou Lu, Wen-Jing Liu, Kai-Li Wang, Yan-Yan Wang, Yi Nan

Abstract

BACKGROUND

Diabetic kidney disease is one of the serious complications of diabetes mellitus, and the existing treatments cannot meet the needs of today's patients. Traditional Chinese medicine has been validated for its efficacy in diabetic kidney disease after many years of clinical application, but the specific mechanism by which it works is still unclear, and the study of the molecular mechanism of the *Nardostachyos Radix et Rhizoma-Rhubarb* drug pair (NRDP)for the treatment of diabetic kidney disease provides a new way of thinking for the research and development of new drugs.

AIM

To investigate the mechanism of the NRDP in diabetic kidney disease by network pharmacology combined with molecular docking, and verified by *in vitro* experiment.

METHODS

The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database was used to screen active ingredient targets of NRDP. Obtain targets for diabetic kidney disease through Genecards, OMIM, and TTD databases. VENNY 2.1 database to obtain diabetic kidney disease and NRDP intersection targets and their Venn diagrams, and Cytoscape 3.9.0 to build a "drug-component-target-disease" network. String database constructs protein interaction networks. KEGG pathway and GO analysis in DAVID database. After selecting the targets and the active ingredients, Autodock software was used to perform molecular docking. In experimental validation using renal tubular epithelial cells (TCMK-1) as the study subject, we used the CCK-8 assay to detect NRDP's effect on cell viability, glucose solution mimics a hyperglycemic environment. Flow cytometry was used to detect the cell cycle and apoptosis. Western Blot was used to detect the protein expression of STAT3, p-STAT3, BAX, BCL-2, CASPASE9, and CASPASE3 in AGE-RAGE signaling pathway.

RESULTS

A total of 10 active ingredients and 85 targets with 111 disease-related signaling pathways were obtained in NRDP. Enrichment analysis of KEGG pathways was performed to determine the core pathway of AGE-RAGE signaling. Molecular docking showed good binding between each active ingredient and its core targets. Experiments *in vitro* showed that NRDP inhibited the viability of TCMK-1 cells, blocked cell cycle progression in the G0/G1 phase, and reduced apoptosis with an increasing drug concentration. Based on the results of Western blot analysis, NRDP differentially downregulates p-STAT3, BAX, CASPASE3, and CASPASE9 protein levels ($P<0.01$ or $P<0.05$), in addition, BAX/BCL-2, p-STAT3/STAT3 expressions are downregulated, while BCL-2 and STAT3 proteins are upregulated($P<0.01$).

CONCLUSION

The NRDP may up-regulate BCL-2 and STAT3 protein expression, and down-regulate BAX, CASPASE3, and CASPASE9 protein expression, thus activating the AGE-RAGE

signaling pathway, inhibiting the vitality of TCMK-1 cells, and reducing the apoptosis of TCMK-1 cells. TCMK-1 cells were blocked in the G0/G1 phase to protect renal tubular epithelial cells induced by high glucose.

Key Words: Nardostachyos Radix et Rhizoma-Rhubarb; Diabetic kidney disease; molecular docking; network pharmacology; experimental validation

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Core Tip: The NRDP may up-regulate BCL-2 and STAT3 protein expression, and down-regulate BAX, CASPASE3, and CASPASE9 protein expression, thus activating the AGE-RAGE signaling pathway, inhibiting the vitality of TCMK-1 cells, and reducing the apoptosis of TCMK-1 cells. TCMK-1 cells were blocked in the G0/G1 phase to protect renal tubular epithelial cells induced by high glucose.

INTRODUCTION

Diabetes Mellitus (DM) is a clinical syndrome mainly characterized by elevated blood sugar caused by genetic factors. Long-term delay and difficult treatment will eventually lead to a series of serious complications, mainly diabetic kidney disease (DKD)[1]. DKD is one of the leading causes of end-stage renal disease (ESRD), which is a leading cause of kidney failure. According to the epidemiological survey data released by the International Diabetes Federation (IDF), the global incidence of DM is 9.3% [2], among them, 20%-40% develop DKD[3]. With the increase in DKD patients, the treatment of DKD is imminent. The main treatment for DKD in Western medicine is to control blood sugar and improve renal function[4]. Clinical medications are mostly Angiotensin Converting Enzyme Inhibitors (ACEI) and sulfonylureas to improve renal

blood circulation. Despite this, the effect of these medications on relieving symptoms and reducing the disease's progression is not obvious. Hence, we desperately need to find effective drugs or compounds with minimal side effects to treat DKD[5].

As a traditional Chinese medicine in China, Nardostachyos Radix et Rhizoma(Nardostachys jatamansi) belongs to the dried roots and rhizomes of Nardostachys jatamansi, a plant of the Septoria family[6]. Modern pharmacological studies have found that it is effective against brain diseases, heart diseases, spleen diseases, skin diseases, erectile dysfunction, tumors, and other diseases[7]. The chemical components of Nardostachyos Radix et Rhizoma are mainly terpenoids, coumarin, and lignans[8]. Active compounds mansonopsin and Naringin not only relieve cardiac hypertrophy[9] but also have anti-inflammatory, antibacterial, anti-osteoporosis, myocardial protection, anti-malaria, liver protection, anti-apoptosis, anti-tumor, sedative, antihypertensive and anti-oxidative stress effects[10, 11]. Other studies have shown that Nardostachyos Radix et Rhizoma can control blood glucose metabolism, regulate the islet function, and protect the kidney[12]. Rhubarb, which belongs to the dried roots and rhizome of Rheum officinale Baill in the Polygonum family, are widely utilized to cure diverse diseases. Rhubarb prevents the progression of DKD through a variety of mechanisms[13]. Modern pharmacological studies have found that anthraquinone derivatives contained in rhubarb have purgative effects[14]. Anthracene has an antidiarrheal effect. Emodin and rhein have anti-inflammatory, antibacterial, antiviral, anti-oxidative stress, anti-tumor, anti-fibrosis, lipid-regulating, and hypoglycemic effects[15, 16]. Rhubarb tannin improves nitrogen waste metabolism; Rhubarb anthraquinone and rhubarb anthraquinone glucoside can inhibit mesangial cell growth, improve renal tubular function, and protect the kidney [17]. Rhein has antitumor effects[18]. In addition, it has the functions of hemostasis, antiviral, antibacterial, liver protection[19], gallbladder protection, stomach protection[20], and kidney protection. In the treatment of DKD, rhubarb can reduce uremic toxin levels, regulate intestinal flora[14], and delay renal interstitial fibrosis. However, the drug targets and molecular mechanisms of NRDP for the treatment of DKD have not been

clarified. Therefore, we investigated the specific drug targets and molecular mechanisms of NRDP for the treatment of DKD from the study of network pharmacology combined with pharmacology.

DM and aberrant renal function are the causes of DKD. While the precise etiology remains unknown, certain research has demonstrated that Advanced Glycation End Products (AGEs) formation is essential to the development of DKD. When its receptor RAGE is activated, other associated pathways are impacted, which increases oxidative stress and inflammation in renal cells, encouraging apoptosis and exacerbating the progression of DKD[21]. Traditional Chinese medicine is often used to treat chronic diseases. In the state of high glucose, the AGE-RAGE pathway will be activated to increase kidney damage. Studies have shown that traditional Chinese medicine monomers and compounds can regulate PI3K-AKT, NF-κB, JAK/STAT, and other pathways. Reduce oxidative stress, cell apoptosis, and inflammation, thereby improving kidney damage and delaying the course of DKD[22]. We found that the research field of NRDP treating DKD with a target point has not been involved, so we chose this direction as the research target, analyzed its active components and targets, and investigated how NRDP treats DKD experimentally, providing research ideas and theoretical basis for further exploration of the material basis and mechanism of action of NRDP treating DKD.

Traditional Chinese medicine compounds exhibit multi-target, multi-component, and multi-pathway actions that are consistent with network pharmacology. In this study, we employed network pharmacology and experimental verification to confirm the mechanism of action of the compound on DKD. We searched for drug and disease targets using the TCMSP, Gene Cards, OMIM, and TTD databases, and then identified the core target pathway through the PPI protein interaction network. Using NRDP components, we conducted GO and KEGG enrichment analyses, as well as molecular docking. Finally, we conducted an experimentally verified method to prove the predictions made on the mechanism of NRDP in DKD. The aim is to provide new ideas and methods for the subsequent treatment of DKD with traditional Chinese medicine.

MATERIALS AND METHODS

NRDP for the acquisition of active ingredients and targets

The TCMSP database analysis platform (https://tcmsp-e.com/) was searched for Nardostachyos Radix et Rhizoma and Rhubarb, oral bioavailability tests were conducted for active ingredients in drugs and their corresponding targets (oral availability, OB) ≥ 30%, drug-like properties (drug-likeness, DL) ≥ 0.18, and then UniProt (https://www.uniprot.org/) database was used to translate the targets into gene names.

Obtain DKD targets

In GeneCards (http://www.genecards.org/), OMIM (http://omim.org/), and TTD (https://db.idrblab.net/ttd/), "Diabetic Kidney Diseases" and "Diabetic Nephropathy" were searched as keywords, and the DKD targets were obtained.

Obtain intersection targets for NRDP and DKD

Venny2.1.0 platform (https://bioinfogp.cnb.csic.es/tools/venny/) was used to obtain the common targets between the NRDP and DKD.

Construction of "component-target-disease" network

To visualize the results, a "component-target-disease" network was constructed using Cytoscape 3.9.0 with the active ingredients and targets of NRDP and DKD.

Construction of protein-protein interaction (PPI) network

PPI network diagrams were constructed by importing the common targets of selected NRDP and DKD into the STRING 11.5 database (https://cn.string-db.org/), and the species was set as "Homo sapiens". The minimum interaction threshold is set as "highest confidence" (> 0.9), to hide isolated nodes, and the rest of the settings were set as default. To obtain protein interaction data, download the TSV file and import Cytoscape software, use the Network Analyzer plug-in to analyze network characteristics, and screen core targets in the PPI network according to the degree of nodes.

Compositions-targets network diagram construction

Cytoscape software was used to construct a network for common targets and NRDP active components. To analyze the characteristics of the network, the Network Analyzer plug-in was used, and the interaction between NRDP active components and core targets was analyzed according to the degree of nodes.

GO and KEGG enrichment analysis

Intersection targets were imported to the DAVID database (https://david.ncifcrf.gov/) for GO (gene ontology) function and KEGG (Kyoko encyclopedia of genes and genomes) pathway enrichment analysis. P-value< 0.01 and FDR<0.01 are the conditions and the entries that meet the screening. With the help of online data analysis, the visualization platform - microscopic letter (http://www.bioinformatics.com.cn/) resulted in visualization.

Construction of component-target network diagram in the signal pathway

The target on the core pathway obtained after KEGG enrichment is constructed using the STRING11.5 database (same construction conditions as before). TSV file of PPI is downloaded and imported into Cytoscape software. After analysis by the Network Analyzer plug-in, A component-target network diagram about the pathway was reconstructed with the active components of NRDP. According to the degree value of the node, NRDP components interacted with targets on the pathway.

Molecular Docking

NRDP medicine mol2 structures were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and downloaded the core target protein 3D structure through the PDB database (https://www.rcsb.org/). Then, the water molecules and small molecular ligands of proteins were removed using Pymol 2.4.0 software, and AutoDock 1.5.7 software was imported for hydrogenation. Molecular docking of receptor and ligand was performed and their binding activity was evaluated.

Cell experiment verification

Cells

TCMK-1(Renal tubule epithelial cells), purchased from BeNa Culture Collection (No. BNCC339820).

Drugs and reagents

NRDP: Preparation Center, Affiliated Hospital of Traditional Chinese Medicine, Ningxia Medical University.

Cell culture

TCMK-1 cells were cultured with complete medium (DMEM high-glucose medium +10%FBS+1% penicillin mixture) in an incubator of 37°Cand 5% CO_2. A microscope was used to observe cell growth, and 80% of the cells were confluent for passage.

The half inhibitory concentration of NRDP was detected by the CCK8 method

TCMK-1 cells at the logarithmic growth stage were added to pancreatic enzyme for digestion, and cell suspension was prepared. Counting was performed under 20 times microscope. The control group (complete cell culture medium), Model group (60mmol•L-1 high glucose culture medium), and 5×10^3 cells were inoculated per well into 96-well plates for the NRDP group, each group had 5 repeat Wells. The drug was diluted multiple times according to the drug concentration gradient, 100ul was added to each well, and the cells were treated for 24h. At the end of the drug intervention, Incubation with CCK8 was performed for 1h under no light conditions by adding 10ul to each well. The enzyme marker was opened and the wavelength parameter was set to 450nm to detect cell optical density (OD) for statistical analysis.

The effect of NRDP on the TCMK-1 cell cycle induced by high glucose was determined by flow cytometry

TCMK-1 cells at logarithmic growth stage were divided into 5 groups: Control group (complete culture medium), Model group (60mmol•L-1 high-glucose culture medium), low-dose group (high-glucose culture medium +4mg•ml-1NRDP), medium-dose group (high-glucose culture medium +7mg•ml-1NRDP), high-dose group (high-glucose culture medium +10mg•ml-1NRDP). Three replicates were performed in each group and inoculated into 6-well plates at a density of 1.0×10^5 cells/well. Following 24 h of culture in the incubator, serum-free medium was added to each group for 12 h of

intervention. The digested cells were gathered, fixed for an overnight period, and pre-cooled by adding 70% ethanol. The cell cycle kit (KeyGEN Biotech, China) was followed by adding the reagents and incubating them for 30 minutes before detecting the results using CytoFLEX flow cytometer (Beckman Coulter, US). Statistics were used to analyze the outcomes.

Flow cytometry was used to detect the effect of NRDP on the apoptosis of TCMK-1 cells induced by high glucose

TCMK-1 cells at the logarithmic growth stage were divided into 5 groups (the cell group was the same as the cell cycle experiment group) and three replicates were performed in each group. It was inoculated into 6-well plates at a density of 1.0×10^5 cells/well. For 24 h following culture in the incubator, each group was exposed to the corresponding culture medium. Digested cells were collected and the corresponding reagents were added according to the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (KeyGEN Biotech, China)instructions. The results were detected by CytoFLEX flow cytometry(Beckman Coulter, US) after 15 minutes and then statistically analyzed.

Protein expression was detected using Western blot

TCMK-1 cells at the logarithmic growth stage were divided into three groups: control group, model group, and medium dose group (7mg•ml-1NRDP), and three replicates were performed in each group. Collect digestive cells to join 200ul RIPA cracking fluid, according to the total protein extraction kit (KeyGEN Biotech, China) instruction, and the extracted proteins in protein content determination. SDS-PAGE electrophoresis was carried out according to the quantitative results of protein. After mold rotation and sealing, TBST was used for cleaning 3 times, and TBST diluted primary antibody was placed at 4℃overnight. After constant temperature reaction for 1h, incubated in the secondary antibody for 1h. After TBST cleaning 3 times, the luminous liquid was added for exposure, and the Chemidoc instrument (Ge1Doc XR+, BIO-RAD, US) was used for acquiring chemiluminescence. Statistical analysis was conducted using Image J software to determine gray values.

Statistical Methods

GraphPad Prism 8.0.2 software was used for statistical analysis and one-way analysis of variance was used to compare groups. SNK test was used for homogeneity of variances, and Tamhane'sT test was used for heterogeneity of variances. Taking $P<0.05$ indicates statistically significant results.

RESULTS

Chemical constituents and targets of NRDP

A total of 15 active ingredients were obtained from the TCMSP database, including 5 active ingredients from Nardostachyos Radix et Rhizoma and 10 active ingredients from Rhubarb. After removing duplicate targets, 43 targets of Nardostachyos Radix et Rhizoma, 69 targets of Rhubarb, and 85 GRDP targets were obtained. (see Figure 2A).

Drug-disease Intersection targets and Venn Diagram

Based on GeneCards (https://www.genecards.org/), OMIM (https://omim.org/), DrugBank (https://go.drugbank.com/), TTD database (http://db.idrblab.net/ttd/) a total of 7046 relevant targets for DKD were screened (see figure 2A). A Venn diagram was drawn for 73 intersection targets between NRDP and DKD (see Figure 2B).

Construction of drug-disease-active ingredient-target network

The NRDP active ingredients and common targets were imported into Cytoscape 3.9.0 software to obtain a visualized regulatory network diagram (see Figure 2C). The nodes in the diagram include drug, disease, active ingredient, and target, where the edges indicate that there is an interrelationship between them. Orange represents diseases and drugs. The fuchsia represents the co-interacting active ingredient of NRDP-disease. Light blue represents the active ingredient of Nardostachyos Radix et Rhizoma. The light yellow represents the active ingredient of Rhubarb. Light purple represents gene targets for NRDP-disease co-action. Pink represents the target genes for Nardostachyos Radix et Rhizoma alone action. Light orange represents target genes for Rhubarb acting alone. This network diagram visualizes that NRDP drugs work through multiple components and targets in the treatment of DKD.

Protein interaction network diagram

Using the STRING database, we obtained the protein interaction network diagram of NRDP and DKD (see Figure 2D). There are 54 nodes and 310 edges, and the nodes represent the protein.

PPI network analysis

The TSV file of the above PPI Network diagram was downloaded, and the Network Analyzer plug-in in Cytoscape was used to analyze the network characteristics, including 54 nodes and 178 edges. Nodes with a degree median greater than 13 are carded out, to obtain the PPI network, the core target of NRDP for DKD treatment. The targets were TP53, STAT3, HSP90AA1, JUN, RELA, ESR1, CCND1, MYC, CDKN1A, NR3C1 and CDK1. The results were visualized and output, as shown in Figure 2E.

Composition and target network analysis

A network diagram was constructed between PPI targets and NRDP active components. Through the analysis of the Network Analyzer plug-in in Cytoscape software, the interaction between NRDP active components and 54 targets and their degree values were obtained. Nodes with a median of greater than 13 degrees were found. Seven main components were obtained, including aloe-emodin, cryptotanshinone, EUPATIN, rhein, and acacetin, as shown in Figure 2F.

GO Analysis and KEGG Analysis

A total of 1528 biological process (BP) entries were obtained after GO enrichment analysis, which mainly involves response to oxygen-containing compound and response to organic cyclic compound, cellular response to chemical stimulus, cellular response to oxygen-containing compound, *etc.* 107 items of cell composition (CC), it contains the membrane raft, cytoplasmic part, an integral component of the presynaptic membrane and plasma membrane, extracellular exosome and cytosol. There are 115 molecular function (MF) entries, mainly involving enzyme binding, protein domain-specific binding, and transcription factor activity. Transcription factor activity, direct liNRDPnd regulated sequence-specific DNA binding, *etc.* The first 10 enrichment results of each group were plotted (see Figure 3A). The KEGG pathway enrichment analysis

identified 111 pathways. The analysis identified 131 pathways. The first 20 pathways were mapped (see Figure 3B). The pathways involved include Pathways in cancer, PI3K-AKT signaling pathway, p53 signaling pathway pathway, AGE-RAGE signaling pathway in diabetic complications and apoptosis signaling pathway are important pathways for the treatment of DKD under NRDP. The AGE-RAGE signaling pathway is the core pathway. The component-target Network diagram in the signal pathway was constructed with NRDP active components through the analysis of gene PPI network database STRING11.5 and Network Analyzer plug-in of Cytoscape software, and the results were visualized (see Figure 3C) and the signal pathway diagram (see Figure 3D).

Molecular Docking

To further analyze the feasibility of NRDP for the treatment of DKD. The core proteins TP53, STAT3, HSP90AA1, JUN, RELA, ESR1, and CCND1, which were ranked in the top seven of the degree value, were molecularly docked with the active components of NRDP. The PDB ID of ESR1 was 6CHW, RELA was 3CBQ, TP53 was 3DCY, HSP90AA1 was 1BYQ, JUN was 2P33, CCND1 was 2VTH, STAT3 was 6NJS. Pymol software was used to visualize the docking results (Figure 4A). Binding activity was evaluated according to docking scores: scores<-4.25 kcal·mol-1 indicated that the two had certain binding activity, fraction<-5.0 kcal·mol-1 indicated good binding activity, and fraction<-7.0 kcal·mol-1 indicates strong binding activity. Through ChiPlot (https://www.chiplot.online/#Heatmap) online tools for the visualization output (see Figure 4B). The binding energy of 10 active ingredients with 7 core target proteins was less than -5.0 kcal·mol-1, among which the binding energy of STAT3 and (-) -Catechin was the smallest, and its binding energy was -9.3 kcal·mol-1.

The median inhibitory concentration of NRDP

To determine the inhibition rate of each group, the OD values from the 24-hour experiment were added to an inhibition rate calculation algorithm. With GraphPad Prism 8.0.2, half-inhibitory doses of TCMK-1 cells were fitted (see Figure 5A). According to the experimental results, its IC_{50} value was 7.84mg•ml-1, so we determined that the half inhibitory concentration was 7mg•ml-1, and the optimal

administration concentration was 4mg•ml-1 at low dose, 7mg•ml-1 at medium dose and 10mg•ml-1 at high dose. CCK-8 assay showed that compared with the control group (CON), the cell viability of the model group (MOD) was significantly increased (P<0.01). Compared with the model group (MOD), TCMK-1 cell viability decreased after NRDP intervention (P<0.01), and the decreasing trend showed dose-dependent tolerance. The higher the NRDP dose, the more obvious the decline in TCMK-1 cell viability (see Figure 5B).

The effect of NRDP on the TCMK-1 cell cycle induced by high glucose was determined by Flow Cytometer

After 24h of NRDP intervention, flow cytometer was used to determine the cell cycle of TCMK-1 cells. Compared to the control group (CON), model group (MOD) cells showed an increase in G0/G1 phase. (P<0.01), compared with the model group, the proportion of the G0/G1 phase increased and the S phase decreased after NRDP intervention (P<0.01). It was shown that NRDP could block TCMK-1 cells in the G0/G1 phase, as shown in Figure 5C. G0/G1 phase CON 60.1±0.70, MOD 40.23±1.07, NRDP-L 45.55±1.23, NRDP-M 48.93±0.75, NRDP-H 61.04±1.66.

Flow Cytometer was used to detect the effect of NRDP on the apoptosis of TCMK-1 cells induced by high glucose

Apoptosis of TCMK-1 cells was detected by AV-PI double staining and flow cytometer after 24h of NRDP intervention. Compared with the control group (CON), the apoptosis ratio of TCMK-1 cells in the model group (MOD) was significantly increased (P<0.01); Compared with the MOD group, the percentage of apoptosis in NRDP dose groups was decreased (P<0.01), and with the increase of NRDP concentration, the apoptosis ratio of TCMK-1 cells decreased more significantly, as shown in Figure 5D. The data are as follows: CON 7.17±0.168, MOD 19.32±1.975, NRDP-L 15.55±0.257, NRDP-M 12.80±0.773, NRDP-H 10.18±0.523.

Western blot was used to detect the expression of core proteins

Western blot was used to detect the expression of core protein in TCMK-1 cells after NRDP intervention. The expression of p-STAT3, BAX, CASPASE3, CASPASE9,

BAX/BCL-2, and p-STAT3/STAT3 proteins was increased in the MOD group compared with the CON group ($P<0.01$). The expression of BCL-2 and STAT3 proteins was decreased ($P<0.01$). After intervention with NRDP, the expression of p-STAT3, BAX, CASPASE3, CASPASE9, BAX/BCL-2 and p-STAT3/STAT3 proteins was decreased ($P<0.01$ or $P<0.05$). The expression of BCL-2 with STAT3 proteins was increased ($P<0.01$) as shown in Figure 6.

DISCUSSION

NRDP for the acquisition of active ingredients and targets

The TCMSP database analysis platform (https://tcmsp-e.com/) was searched for Nardostachyos Radix et Rhizoma and Rhubarb, oral bioavailability tests were conducted for active ingredients in drugs and their corresponding targets (oral availability, OB) ≥ 30%, drug-like properties (drug-likeness, DL) ≥ 0.18, and then UniProt (https://www.uniprot.org/) database was used to translate the targets into gene names.

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Obtain common targets for NRDP and DKD

Using the Venny2.1.0 platform (https://bioinfogp.cnb.csic.es/tools/venny/) the common targets were obtained from the intersection between the NRDP drug targets and the DKD targets.

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The effect of NRDP on the TCMK-1 cell cycle induced by high glucose was determined by flow cytometry

TCMK-1 cells at logarithmic growth stage were divided into 5 groups: Control group (complete culture medium), Model group (60mmol•L-1 high-glucose culture medium), low-dose group (high-glucose culture medium +4mg•ml-1NRDP), medium-dose group (high-glucose culture medium +7mg•ml-1NRDP), high-dose group (high-glucose culture medium +10mg•ml-1NRDP). Three replicates were performed in each group and inoculated into 6-well plates at a density of 1.0×10^5 cells/well. Following 24 h of culture in the incubator, serum-free medium was added to each group for 12 h of intervention. The digested cells were gathered, fixed for an overnight period, and pre-cooled by adding 70% ethanol. The cell cycle kit (KeyGEN Biotech, China) was followed by adding the reagents and incubating them for 30 minutes before detecting the results using CytoFLEX flow cytometer (Beckman Coulter, US). Statistics were used to analyze the outcomes.

Flow cytometry was used to detect the effect of NRDP on the apoptosis of TCMK-1 cells induced by high glucose

TCMK-1 cells at the logarithmic growth stage were divided into 5 groups (the cell group was the same as the cell cycle experiment group) and three replicates were performed in each group. It was inoculated into 6-well plates at a density of 1.0×10^5 cells/well. For 24 h following culture in the incubator, each group was exposed to the corresponding culture medium. Digested cells were collected and the corresponding reagents were added according to the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (KeyGEN Biotech, China)instructions. The results were detected by CytoFLEX flow cytometry(Beckman Coulter, US) after 15 minutes and then statistically analyzed.

Protein expression was detected using Western blot

TCMK-1 cells at the logarithmic growth stage were divided into three groups: control group, model group, and medium dose group (7mg•ml-1NRDP), and three replicates were performed in each group. Collect digestive cells to join 200ul RIPA cracking fluid, according to the total protein extraction kit (KeyGEN Biotech, China) instruction, and the extracted proteins in protein content determination. SDS-PAGE

electrophoresis was carried out according to the quantitative results of protein. After mold rotation and sealing, TBST was used for cleaning 3 times, and TBST diluted primary antibody was placed at 4℃overnight. After constant temperature reaction for 1h, incubated in the secondary antibody for 1h. After TBST cleaning 3 times, the luminous liquid was added for exposure, and the Chemidoc instrument (Ge1Doc XR+, BIO-RAD, US) was used for acquiring chemiluminescence. Statistical analysis was conducted using Image J software to determine gray values.

Statistical Methods

GraphPad Prism 8.0.2 software was used for statistical analysis and one-way analysis of variance was used to compare groups. SNK test was used for homogeneity of variances, and Tamhane'sT test was used for heterogeneity of variances. Taking $P<0.05$ indicates statistically significant results.

CONCLUSION

In this study, NRDP was used as a research object to investigate its mechanism of action on DKD using network pharmacology. The results showed that NRDP had a therapeutic effect on DKD, with 10 active ingredients involving 85 targets. The GO analysis, KEGG analysis, and network interaction software analysis revealed that NRDP may act on DKD through the AGE-RAGE signaling pathway. Our *in vitro* cell experiments confirmed the prediction that NRDP significantly inhibited TCMK-1 proliferation and promoted cell cycle arrest at the G0/G1 phase, which reduced the apoptosis of TCMK-1 cells in a dose-dependent manner. The results of the Western blot analysis indicate that NRDP intervention led to up-regulation of BCL-2 and STAT3 protein expressions, and down-regulation of p-STAT3, BAX, CASPASE3, and CASPASE9 protein expressions. Additionally, the down-regulation of BAX/BCL-2 and p-STAT3/STAT3 protein expressions was observed. These findings suggest that NRDP is an effective treatment for DKD. NRDP protects renal tubular epithelial cells from high glucose-induced damage by controlling the AGE-RAGE signaling pathway.

AGEs are a group of complex molecules that form through non-enzymatic reactions between proteins or lipids and glucose or other carbohydrate derivatives. Its receptor RAGE is a multi-ligand receptor belonging to the immunoglobulin superfamily and is expressed in a wide range of tissues, including the vascular system, lung, heart, endothelial, and nervous tissues [23]. When combined, they form a key pathophysiological process associated with the occurrence and progression of many diseases, especially diabetes complications. Elevated blood levels of AGEs from hyperglycemia interact with RAGE to activate many downstream effectors, including the JAK/STAT pathway, which in turn activates transcription factors like STAT3 over time[24]. Increases the inflammatory response, which further exacerbates DKD[25]. Tang D[26] showed through network pharmacology that the AGE-RAGE pathway is the most important pathway for Coptis Jiedu decoction to fight DKD, and *in vivo* experiments verified that Coptis Jiedu decoction can improve glucose and lipid metabolism disorder and kidney injury by regulating the AGEs-RAGE-AKT-Nrf2 pathway in db/db mice, thus playing a protective role in DKD. Hou Biyu[27] showed that salvianolic acid A inhibited AGEs-induced actin cytoskeletal rearrangement through the AGEs-RAGE-Rhoa-Rock pathway, restored glomerular endothelial permeability, weakened AGEs-induced oxidative stress, restored glomerular endothelial function, alleviated renal structural deterioration, and effectively improved early DKD. The changes in the expression of relevant proteins after NRDP intervention in this experiment showed that the drug alleviated DKD symptoms to some extent.

Numerous studies have demonstrated that the production of AGEs linked to hyperglycemia is a key factor in the pathophysiology of DKD. The receptor for AGEs (RAGE) binds to its ligands, inducing oxidative stress and chronic inflammation in renal tissue, ultimately resulting in renal dysfunction. AGEs can alter the extracellular matrix (ECM) by involving cell surface receptors and producing proinflammatory cytokines. RAGE and its ligands promote angiogenesis, cell migration, proliferation, invasion, and metastasis by limiting apoptotic cell death[28]. Studies have shown that AGEs and their receptor RAGE can induce apoptosis in different cell types. The

propagation of apoptosis through the AGE-RAGE signaling pathway involves the cascade reaction of the pro-apoptotic factor, which prompts the apoptotic signal to activate the apoptotic factor CASPASE-3[29] and initiates the occurrence of apoptosis. Under the influence of certain receptors and factors, the endogenous apoptotic pathway is activated and regulated by the BCL-2 protein, which directly activates CASPASE9. The CASPASE cascade can activate CASPASE3 during apoptosis induced by death receptors and DNA damage, producing intracellular signals that act on cellular targets, ultimately leading to programmed cell death[30]. Previous studies have demonstrated that RAGE expression regulates apoptotic death receptors and mitochondrial pathways by controlling the expressions of pro-apoptotic CASPASE-3, CASPASE-9, and anti-apoptotic BCL-2. BAX is a proapoptotic protein, and BCL-2, as a regulatory protein of apoptosis, can form a Bax-Bcl-2 heterodimer when it binds to active BAX protein in the cytoplasm, which can play a role in reducing apoptosis. Reducing the activity of the BAX protein can also negatively regulate apoptosis. The amount of apoptosis can be determined by the degree of binding between BAX and BCL-2 protein. Reducing the activity of BAX and promoting the binding of BCL-2 to BAX protein can reduce apoptosis[31]. Our study found that the expression of apoptosis-related proteins in TCMK-1 cells was detected after the intervention of NRDP. The expression of BAX, CASPASE3, and CASPASE9 proteins showed a downregulation trend, while the expression of BCL-2 protein showed an increase. This may be due to the activation of BCL-2 expression by NRDP. The inhibition of BAX protein activity resulted in a weakened CASPASE family cascade and reduced apoptosis of renal tubular epithelial cells. This illustrates the pharmacological effect of NRDP in treating DKD.

In summary, NRDP may prevent TCMK-1 cells from proliferating and reduce cell death by controlling the relevant proteins on the AGE-RAGE signaling pathway, thereby protecting the function of intrinsic kidney cells during high glucose levels. Currently, there are numerous studies on the pathogenesis of DKD, which can be summarized as the influence of metabolic, inflammatory, hemodynamic, and fibrotic factors. Many experts and scholars have explored the treatment of DKD. Some

treatments targeting specific pathogenic mechanisms are often used in clinical and experimental studies. Combination therapies involving two or more drugs have been found to have the potential to treat DKD. For instance, combining ERA with SGLT2 inhibitors has shown promise[32]. The present study also validates the efficacy of an herbal combination for treating DKD, providing a preliminary possibility for future exploration of new targets of traditional Chinese medicine combined with other inhibitors and drugs for treating DKD. However, this study was limited to *in vitro* cellular experiments due to current energy and funding constraints. Our group's research on treating DKD with traditional Chinese medicine is ongoing, and we plan to incorporate high-throughput histological methods for further validation in the future. In the future, we will be using high-throughput genomics methods for the validation and identification of a safe and effective clinical treatment for DKD, which will improve the prognosis and quality of life of patients.

CONCLUSION

In this study, TCMK-1 cells were treated with varying concentrations of NRDP to intervene in a hyperglycemic environment. The results indicate that NRDP can regulate the cell cycle of TCMK-1 cells by blocking them in the G0/G1 phase, affecting the process from the late stage of DNA synthesis to the completion of mutism, and reducing apoptosis in a dose-dependent manner. Additionally, NRDP may up-regulate the expression of BCL-2 and STAT3. The expression of p-STAT3, BAX, CASPASE3, and CASPASE9 proteins were down-regulated, as well as BAX/BCL-2 and p-STAT3/STAT3. Consequently, the impaired AGE-RAGE signal axis had a greater impact on the body during high glucose conditions, and the high glucose environment had a protective effect on renal tubular epithelial cells. This lays the foundation for the search for safe and effective drugs to treat DKD.

ARTICLE HIGHLIGHTS

Research background

The TCMSP database was used to screen active ingredient targets of NRDP. Obtain targets for diabetic kidney disease (DKD) through Genecards, OMIM, and TTD databases. VENNY 2.1 database to obtain DKD and NRDP intersection targets and their Venn diagrams, and Cytoscape 3.9.0 to build a "drug-component-target-disease" network. String database constructs protein interaction networks.KEGG and GO enrichment analysis in DAVID database. Autodock software was used to perform molecular docking. In experimental validation using TCMK-1 as the study subject. Using flow cytometry to detect the cell cycle and apoptosis.Western Blot assay protein expression on the AGE-RAGE signaling pathway.

Research motivation

Network pharmacology and molecular docking techniques were used to predict the target of NRDP in the treatment of DKD. Then *in vitro* cell experiments were used to verify the accuracy of the prediction. It provides a theoretical and preliminary experimental basis for subsequent clinical medication.

Research objectives

In-depth study of the specific mechanism of NRDP treatment of DKD provides new ideas and methods for safe and effective treatment of DKD in the future.

Research methods

Diabetic kidney disease is one of the serious complications of diabetes mellitus, and the existing treatments cannot meet the needs of today's patients. Traditional Chinese medicine has been validated for its efficacy in diabetic kidney disease after many years of clinical application, but the specific mechanism by which it works is still unclear, and the study of the molecular mechanism of the Nardostachyos Radix et Rhizoma-Rhubarb drug pair (NRDP)for the treatment of diabetic kidney disease provides a new way of thinking for the research and development of new drugs.

Research results

In order to treat DKD more effectively and reduce the side effects of drugs, find a natural, effective and safe drug for future clinical treatment of DKD

Research conclusions

To explore the mechanism of the Nardostachyos Radix et Rhizoma-Rhubarb drug pair (NRDP) in diabetic kidney disease by network pharmacology combined with molecular docking, and verified by *in vitro* experiment. To provide the theoretical and experimental basis for future clinical drug use.

Research perspectives

A total of 10 active ingredients and 85 targets with 111 disease-related signaling pathways were obtained in NRDP. Enrichment analysis of KEGG pathways was performed to determine the core pathway of AGE-RAGE signaling. Experiments *in vitro* showed that NRDP inhibited the viability of TCMK-1 cells, blocked cell cycle progression in the G0/G1 phase, and reduced apoptosis with an increasing concentration of the drug. Based on the results of Western blot analysis, NRDP differentially downregulates p-STAT3, BAX, CASPASE3, and CASPASE9 protein levels(P<0.01 or P<0.05), in addition, BAX/BCL-2, p-STAT3/STAT3 expressions is downregulated, while BCL-2 and STAT3 proteins are upregulated(P<0.01).

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