Original Article

New indication of Chuankezhi injection for steroid-resistant focal segmental glomerulosclerosis and its mechanism of action

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Background: Chuankezhi (CKZ) injection is a traditional Chinese medicine (TCM) injection extracted from Chinese herbs *Epimedium sagittatum* (Yin Yang Huo) and *Morinda officinalis* (Bai Ji Tian). Studies have shown that CKZ has a positive effect on improving diabetic nephropathy and regulating immune function. Focal segmental glomerulosclerosis (FSGS) is a kind of refractory nephropathy, which has been confirmed as closely associated with immunity. Whether CKZ is effective against FSGS and how it works warrant further study. This study aimed to verify the efficacy of CKZ in rats with steroid-resistant (SR) FSGS and explore its mechanism of action.

Methods: We established an SR FSGS model in male Sprague Dawley (SD) rats by injecting adriamycin into the tail vein. Based on group intervention and comparison, the primary efficacy parameters of FSGS were observed, including general condition, 24-hour urine protein, serum albumin, cholesterol, triglyceride, and renal pathological changes. Network pharmacological analysis and molecular docking were used to predict the mechanism of action of CKZ. Finally, we used quantitative polymerase chain reaction (qPCR) and western blot (WB) to detect messenger RNA (mRNA) expression and protein phosphorylation at specific targets in rat kidney tissue to validate the predicted results.

Results: Intramuscular injection of CKZ had a dose-dependent effect in SR FSGS model rats, including lowering urine protein, increasing serum albumin, lowering cholesterol and triglyceride, and treating pathological lesions in the kidney. Network pharmacological analysis and Molecular docking revealed that 5 active components (Icariin, Icariside II, Epimedin C, Icaritin, and Noricaritin) might be the critical components. The findings also revealed that Akt was perhaps the critical target gene, the PI3K-Akt signaling pathway was perhaps the critical pathway, and reversible protein phosphorylation was probably the critical biological process. The qPCR and WB analyses showed that CKZ significantly increased the relative mRNA expression and protein phosphorylation of PI3K and Akt, respectively.

Conclusions: This study showed that intramuscular injection of CKZ has a significant therapeutic effect in SR FSGS rats, which may be associated with the activation of PI3K-Akt signaling by CKZ.

Keywords: Chuankezhi (CKZ) injection; focal segmental glomerulosclerosis (FSGS); new indication; mechanism of action; PI3K-Akt signaling

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Introduction

Chuankezhi (CKZ) injection is a traditional Chinese medicine (TCM) injection extracted from the Chinese herbs *Epimedium sagittatum* (Yin Yang Huo) and *Morinda officinalis* (Bai Ji Tian), which has been officially approved by the National Medical Products Administration of China (State medical license No. Z20010172). The CKZ injection is administered intramuscularly and has the effects of warming kidney-yang and regulating immunity (1). Studies have shown that CKZ reduces blood lipids and urine protein in patients with diabetic nephropathy (2,3), enhances the regulation of immune function in chronic obstructive pulmonary disease patients (4,5), and may also play a role in reducing toxicity during chemotherapy in cancer patients (6). It has been on the market for 20 years without any severe adverse drug reactions reported; it is a TCM injection with excellent efficacy and safety. Notably, CKZ has been used to treat kidney diseases because of its kidney-yang warming effect; however, to date, there has been little research in this area.

Epimedium sagittatum and *Morinda officinalis* are classic TCM ingredients used to treat kidney-yang deficiency (7). Both herbs (8), and their components (9) have been widely used to treat steroid-resistant (SR) focal segmental glomerulosclerosis (FSGS) by oral administration, and have displayed good auxiliary effects.

The most frequent pathological type of steroid-resistant nephrotic syndrome (SRNS) is FSGS. Due to its high proteinuria and rapid development of renal fibrosis, it is predisposed to end-stage renal disease (ESRD), which causes serious harm to patients (10). Conventional steroid therapy is not effective in FSGS. The current mainstream drug regimen is the use of the immunosuppressant tacrolimus, with further use of rituximab if this is not effective, however, these treatment options still suffer from high individual variability in efficacy, numerous adverse effects, and high costs (11). As of now, it is not precisely clear how FSGS develops. According to TCM theory, the etiology of FSGS and steroid resistance is associated with kidney-yang deficiency (12,13). The damage to podocytes has been found in many modern investigations to be closely related to FSGS (14-17) and involve immune inflammation (18-20).

It may be that SR FSGS is a potential new indication of CKZ, which would hold great clinical value. Further study is warranted to explore whether CKZ is effective against SR FSGS and how it works. Therefore, we applied CKZ, observed its effects on SR FSGS, and explored the potential mechanism of action. This study verified the efficacy of CKZ on adriamycin (ADR) induced SR FSGS model rats, and explored the potential mechanism of action of CKZ by pharmacological network modeling, molecular docking, and detection of relative messenger RNA (mRNA) expression and protein phosphorylation of specific targets in rat kidney tissues. This study provides a solid foundation for further research into this new indication and mechanism of action of CKZ in the treatment of SR FSGS. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1962/rc).

Methods

Preparation of CKZ

We obtained CKZ (batch number: 19112701, 2 mL/bottle) from Guangzhou ImVin Pharmaceutical Co., Ltd. (Guangzhou, China). The CKZ formula consists of 2 herbs, including *Epimedium sagittatum* (the leaves, 55.56%; Yin Yang Huo) and *Morinda officinalis* (the root, 44.44%; Bai Ji Tian).

Firstly, we decocted *Epimedium sagittatum* (500 g) in water 3 times: 1.5 hours for the first time, and 1 hour for the second and third times. The 3 resulting extracts were mixed and filtered. The filtrate was concentrated to a pure cream with a relative density of 1.16–1.24 (50 ℃), then ethanol was added to 70% alcohol content. After stirring and standing overnight, the filtrate was filtered and concentrated to a relative density of 1.06–1.14 (50 ℃). The condensed extract was added to a polyamide column and washed with water. The eluent was discarded. Then, the polyamide column was eluted with 35% ethanol, and the eluent was recycled and filtered. The filtrate was dried under pressure at 80 ℃ to obtain *Epimedium sagittatum* extract.

Secondly, we crushed *Morinda officinalis* (400 g) and sifted it through a 20-mesh sieve to obtain the powder, which was then boiled and concentrated in the same way as *Epimedium sagittatum*. The condensed extract was mixed with ethanol until the alcohol content was 60%. The recycling methods of *Morinda officinalis* extract were the same as *Epimedium sagittatum*, except eluting with 30% ethanol.

Finally, *Epimedium sagittatum* extract, *Morinda officinalis* extract, and sodium chloride (8.5 g) were mixed and added to water to 1,000 mL for injection. The mixture was treated with activated carbon (1 g) and filtered. The filtrate was divided into ampoules and sterilized to obtain CKZ.

Establishment and management of SR FSGS rat model

Animal experiments were performed under a project license (No. TCMF1-2020019) granted by the animal care committee of The First Affiliated Hospital of Guangzhou University of Chinese Medicine, in compliance with the *Regulation on the Administration of Laboratory Animals* (2017 Revision) for the care and use of animals. A protocol was prepared before the study without registration. Guangdong Medical Laboratory Animal Center provided the adult male Sprague Dawley (SD) rats, weighing 300±30 g (Guangzhou, China; quality certificate number: SCXK2018-0002). All animals were housed in a 22±3 ℃ room with 50%±10% humidity and a 12-hour light-dark cycle.

The dose of CKZ was calculated based on the equivalent ratio of humans to rats. According to the instructions for CKZ, the recommended daily dosage of CKZ was 8 mL. The clinical equivalent dosage of CKZ in rats was 0.72 mL/kg/d, based on a dose conversion of a 200 g rat to a 70 kg human. Therefore, the high, medium and low doses of CKZ in this study were 0.72, 0.36, and 0.18 mL/kg/d, respectively. CKZ has been marketed in China for 20 years and is widely used in clinical practice according to the recommended dose in clinical practice, and no serious adverse drug reactions have been reported. Therefore, we believe that the dose selected in this study is safe for the treatment of SR FSGS in the clinical setting.

The dose of prednisone was calculated based on the equivalent ratio of humans to rats. According to the Kidney Disease: Improving Global Outcomes (KDIGO) (21), the recommended daily dosage of prednisone is 2 mg/kg, and the maximum daily total dose does not exceed 60 mg.

The clinical equivalent dosage of prednisone in rats is 12.6 mg/kg·d (the maximum dosage is no more than 5.4 mg), based on a dose conversion of a 200 g rat to a 70 kg human. Therefore, the dose of prednisone in this study was 12.6 mg/kg/d, but the maximum dosage was no more than 5.4 mg.

Following a week of adaptive feeding of 62 male SD rats, 8 rats were chosen as the control group (N) by random number table method, and the remaining 54 rats were used for modeling. The SR FSGS rats were established by injecting ADR hydrochloride into the tail vein. Specifically: ADR 4 mg/kg was injected into the tail vein in the first week; ADR 2 mg/kg was injected into the tail vein in the second week; and the modeling time was 6 weeks in total (22). At the end of 6 weeks, 6 of the 54 modeling rats were randomly selected to receive a 24-hour urine protein test and renal pathological examination. If the 24-hour urine protein test results were more than 100 mg/kg, and the focal segmental sclerosis of glomerulus appeared under the optical microscope, the modeling was deemed successful. After that, the remaining 48 rats were classified into 6 groups (n=8) by random number table method: a model group (M), a prednisone group (P), a high-dose group (CKZ1), a medium-dose group (CKZ2), a low-dose group (CKZ3), and a combined therapy group (CKZ1+P).

Rats in the N and M groups were intramuscularly injected with normal saline daily (1 mL/kg/d) for 6 weeks. The P group was orally administered with prednisone aqueous solution (12.6 mg/kg/d). The CKZ1, CKZ2, and CKZ3 groups were intramuscularly injected with CKZ at dosages of 0.72, 0.36, and 0.18 mL/kg/d, respectively, daily for 6 weeks. The CKZ1+P group was orally administered with prednisone aqueous solution (12.6 mg/kg/d) and intramuscularly injected with CKZ (0.72 mL/kg/d), daily for 6 weeks.

Evaluation of CKZ effect on SR FSGS

General condition

The mental state, activity, reaction speed, hair color, food and water intake, and fecal texture of the rats were observed in comparison with the control group.

Measurement of 24-hour urine protein

We collected 24-hour urine after the intervention. A rat was placed in a metabolic cage with access to water but no food. Urine was centrifuged at 3,000 r/min for 5 minutes, the supernatant was collected in a sterile tube, and 24-hour urine protein was detected by immunoturbidimetry with an automatic biochemical analyzer (Roche cobas 8000, Germany).

Blood biochemistry

After the intervention, blood was collected from the inferior vena cava after isoflurane anesthesia, centrifuged at 3,000 r/min for 15 minutes after standing for 1 hour, the supernatant was collected, and albumin (ALB) was detected by bromocresol green (BCG) method; cholesterol oxidase method was used to measure cholesterol (CHOL); triglyceride (TG) was detected by enzyme method. It was automatically detected by an automatic biochemical analyzer (Roche cobas 8000, Germany).

Pathological observation on the kidney

The left kidney of the rat was excised and washed in saline,

and the renal envelope was peeled off. The upper and lower poles of the kidney were cut into 0.5 cm thick tissues and packed into 5 mL Eppendorf (EP) tubes, add 4% tissue cell fixative, and fix for 48 h at room temperature. The tissues were stained with conventional HE staining and observed 40×, 200×, and 400× under an optical microscope (Nikon Eclipse Ti-SR, Japan). Imaging software (Nikon DS-U3, Japan) was used to collect images.

Identification of CKZ active components and therapeutic targets

Oral availability (OB) testing for TCM active components is often carried out using the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP; https://old.tcmsp-e.com/tcmsp php). However, CKZ is a Chinese patent medicine refined and processed by modern technology, which is used for intramuscular injection and not for oral administration. Therefore, OB screening was not suitable. According to the principle of drug metabolism, after a drug enters the body, the original and metabolic components that can be measured are the main active components of the drug. In this study, the screening of active components of CKZ was based on the original substances and metabolic components, which can be detected after intramuscular injection of CKZ. Pharmaceutical companies have completed the pharmacokinetics study of intramuscular injection of CKZ. To learn about the active components of CKZ, we referred to the relevant research materials published by Guangzhou ImVin Pharmaceutical Co., Ltd.

After obtaining the active components, the therapeutic targets were determined through the SwissTargetPrediciton database (http://swisstargetprediction.ch/).

Obtaining SR FSGS-related targets

We searched 2 public databases for known SR FSGS-related targets using the term "SRNS" and "FSGS": GeneCard (http://www.genecards.org/) and Online Mendelian Inheritance in Man (OMIM; http://www.omim.org/). After merging and removing duplication of the targets from the 2 databases, SR FSGS-related gene targets were obtained.

Network construction and analysis

To uncover the molecular processes of CKZ in the therapy of SR FSGS, 2 kinds of networks were constructed: compound-target (C-T) networks and protein-protein interaction (PPI) networks. Targets and compounds were represented by nodes in the graphical networks, while C-T and PPI were represented by edges.

To obtain the hypothetical CKZ therapeutic targets for SR FSGS, the CKZ and SR FSGS targets were crossed. These prediction targets were then placed into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/), with the confidence level set at 0.9 and the species limited to "Homo sapiens". The PPI data was stored in "TSV" format and integrated into the Cytoscape program (https://cytoscape.org/) to create network diagrams. The CytoNCA plug-in was used in the PPI network to determine degree centrality (DC), betweenness centrality (BC), and closeness centrality (CC), and a gene for the critical therapeutic target was sought.

Enrichment analysis

Gene Ontology (GO) analysis can offer gene and gene product descriptions and annotations that are standardized. Through GO analysis, biological process, cellular component, and molecular function of related genes may be revealed. Gene function analyses and high-level genomic functional data can be obtained in the Kyoto Encyclopedia of Genes and Genomes (KEGG).

To study the biological pathways of pathogenic genes, we utilized the Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.8 (https://david.ncifcrf.gov/) to assess the enrichment of GO and KEGG pathways in the PPI network, with an adjusted P value of 0.05 deemed statistically significant.

Molecular docking

As a strong molecular docking program, SYBYL-X measures the spatial impact, repulsion, hydrogen bond, hydrophobic interaction, and molecular flexibility to provide the total score for a receptor-ligand complex's affinity. The Protein Data Bank (PDB; http://www.rcsb.org/) was used to determine the receptor molecule's matching structural structure for the therapeutic targets. We used SYBYL-X to submit these structures in PDB format. For docking, the chemical structures of active compounds were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov). ChemBio3D Ultra's energy minimizing function was used to obtain the lowest energy conformations, which were saved in MOL2 format and then imported into SYBYL-X. The Docking Suite feature in the SYBYL-X

Table 1 Rat primers for qPCR

Gene	Forward primer	Reverse primer
PI3K	ACCTGGACTTAGAGTGTGCC	TCAGCAGTGTCTCGGAGTTT
Akt	CTGCCCTTCTACAACCAGGA	CATACACATCCTGCCACACG
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

qPCR, quantitative polymerase chain reaction.

program was used to execute molecular docking, and the total score was utilized to assess the degree of molecular docking.

Real-time polymerase chain reaction

Kidney tissue was used to extract total RNA by RNAzol RT (cat. No. qp020, GeneCopoeia, Rockville, MD, USA), and then it was reverse transcribed by fasting RT Kit (ca. No. kr116, Tiangen, Beijing, China). It was quantified by SYBR Select Master Mix Kit (ca. No. 4472908, life) and fluorescence quantitative polymerase chain reaction (qPCR) system. The internal reference gene was applied to *GAPDH*. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of *PI3K*, *Akt* mRNA relative expression. Primer sequences of *PI3K*, *Akt*, and *GAPDH* are shown in *Table 1*.

Western blot

The right kidney of the rat was removed, and the kidney was dissected along the coronal plane after removing the peritoneum. The kidney was then dissected longitudinally, divided into 2 mL lyophilization tubes, coded and labeled, and the kidney was immediately put into a liquid nitrogen tank and then stored in a refrigerator at −80 ℃. Kidney for each group, 100 mg of kidney tissues was weighed, cut into pieces, and placed in 5 mL EP tubes. To obtain total proteins, 20 mg of kidney tissues from each group were lysed using a 200 μL radioimmunoprecipitation assay (RIPA) lysis buffer, which included protease and phosphatase inhibitors. The bicinchoninic acid (BCA) protein quantitative assay kit was used to determine protein concentration. Equal quantities of proteins were electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred to polyvinyl difluoride (PVDF) membranes. Incubation with primary antibodies was performed overnight at 4 ℃ after the membranes had been blocked with 5% nonfat milk. Cell Signaling Technology (Beverly, MA, USA) provided the primary antibodies against PI3K, Akt, phospho-Akt, and GAPDH; Abcam (Cambridge, MA, USA) provided the phospho-PI3K antibody. Anti-rabbit or anti-mouse IgG coupled with horseradish peroxidase (HRP) was the second antibody (1:7,500 dilution). A western blot (WB) detection method with enhanced chemiluminescence (ECL) was used to view the membrane.

Statistical analysis

The software SPSS 23.0 (IBM Corp., Armonk, NY, USA) was used to perform statistical analysis. The data were summarized as mean ± standard error ($\bar{x}$ ± SEM) for all measurements.

If it conformed to the homogeneity of variance (P>0.05) and one-way analysis of variance (ANOVA) showed that there was a difference as a whole (P<0.05), the least significant difference (LSD) method was used for pairwise comparison between groups. When P<0.05, the difference between the 2 groups was considered statistically significant; if it did not conform to the homogeneity of variance, the Tamhane's T2 method was used, and when P<0.05, the difference between 2 groups was considered statistically significant.

Results

CKZ improved ADR-induced SR FSGS

General condition

After modeling, the model rats became increasingly depressed and less responsive, their hair began to appear sparse, withered, and yellow, their dietary intake of water decreased, and their stools gradually became thinner and foul. Compared with the M group, the CKZ groups were significantly improved in all aspects, gradually close to the N group: in good spirits, more responsive, brighter hair, normal intake of food and drink, and well-formed stools.

Measurement of 24-hour urine protein

Compared with the N group, the level of 24-hour urine

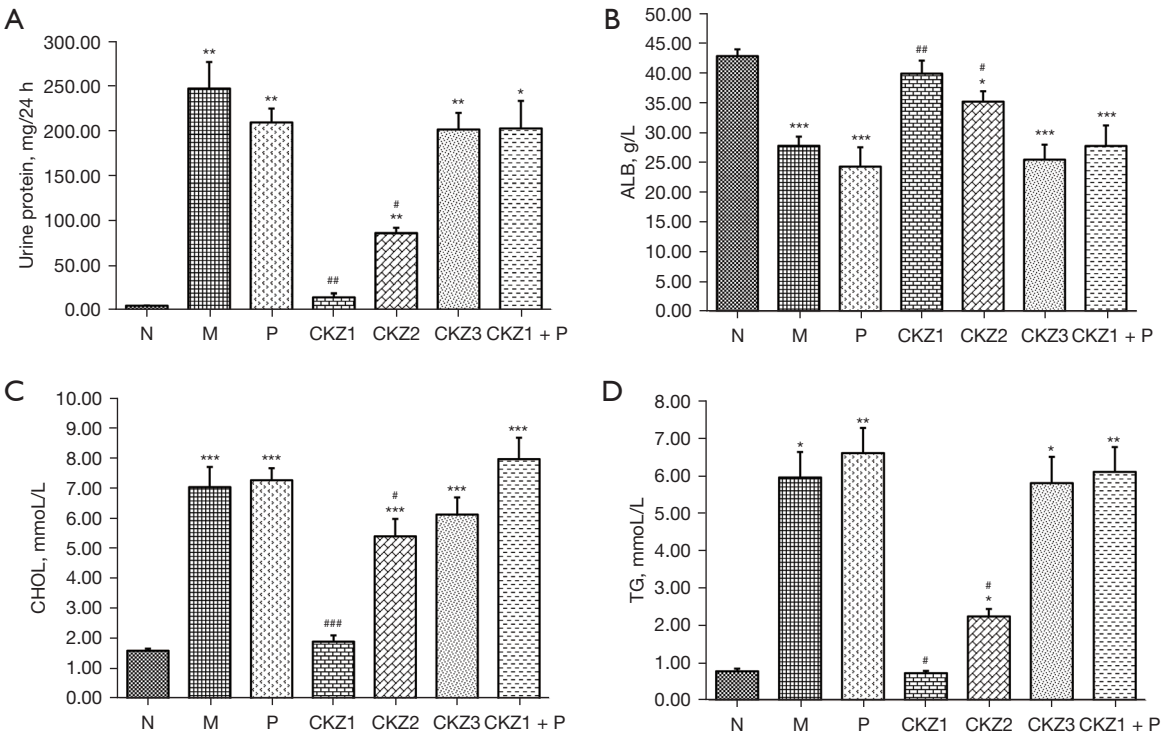


Figure 1 Comparison of 24 h urine protein and blood biochemistry of rats in each group after intervention. N: the control group; M: the model group; P: the prednisone group; CKZ1: the high-dose CKZ group; CKZ2: the medium-dose CKZ group; CKZ3: the low-dose CKZ group; CKZ1+P: the high-dose CKZ + prednisone group. ALB: albumin; CHOL: cholesterol; TG: triglyceride. Data are means ± SEM (n=6). *, P<0.05, **, P<0.01, ***, P<0.001 *vs.* the N group; #, P<0.05, ##, P<0.01, ###, P<0.001 *vs.* the M group, tested by one-way ANOVA. ANOVA, analysis of variance; CKZ, Chuankezhi injection; SEM, standard error of the mean.

proteins in the M group significantly increased (P<0.01). In contrast, CKZ decreased the 24-hour urine proteins in a dose-dependent manner. Besides, the CKZ1+P group could not decrease the 24-hour urine proteins in the SR FSGS rats (*Figure 1A*).

Blood biochemistry

Compared with the N group, the level of ALB in the M group was significantly decreased (P<0.001), the level of CHOL was significantly increased (P<0.001), and TG was significantly increased (P<0.05). In contrast, CKZ increased ALB and decreased CHOL and TG in a dose-dependent manner. Besides, the CKZ1+P group could not improve the above-mentioned blood biochemistry in the SR FSGS rats (*Figure 1B-1D*).

Pathological observation of the kidney

Hematoxylin and eosin (HE)-stained pathological sections of kidneys from rats in each group were examined under an optical microscope in this experiment. The M group had the following pathological changes: glomerular sclerosis, atrophy, lobular mesangial widening, matrix increase, glomerular capillary occlusion, severe balloon adhesion, vitreous degeneration, and sclerotic focus. The renal tubules were dilated, the epithelial cells of renal tubules were partially exfoliated, and protein casts were observed. The epithelial cells of collecting duct were arranged disorderly, and many protein casts were visible. Among the other groups, the renal lesions in the CKZ high-dose group and CKZ medium-dose group were significantly improved. The CKZ high-dose group showed the slightest pathological changes in the kidney. No apparent lesions were found in the glomerulus, renal tubules, or collecting ducts under an optical microscope, which was consistent with the renal pathological manifestations of the N group (*Figure 2*).

Active components and therapeutic targets of CKZ

According to the study of CKZ metabolism in rats (23), the active components of CKZ comprise 11 metabolic

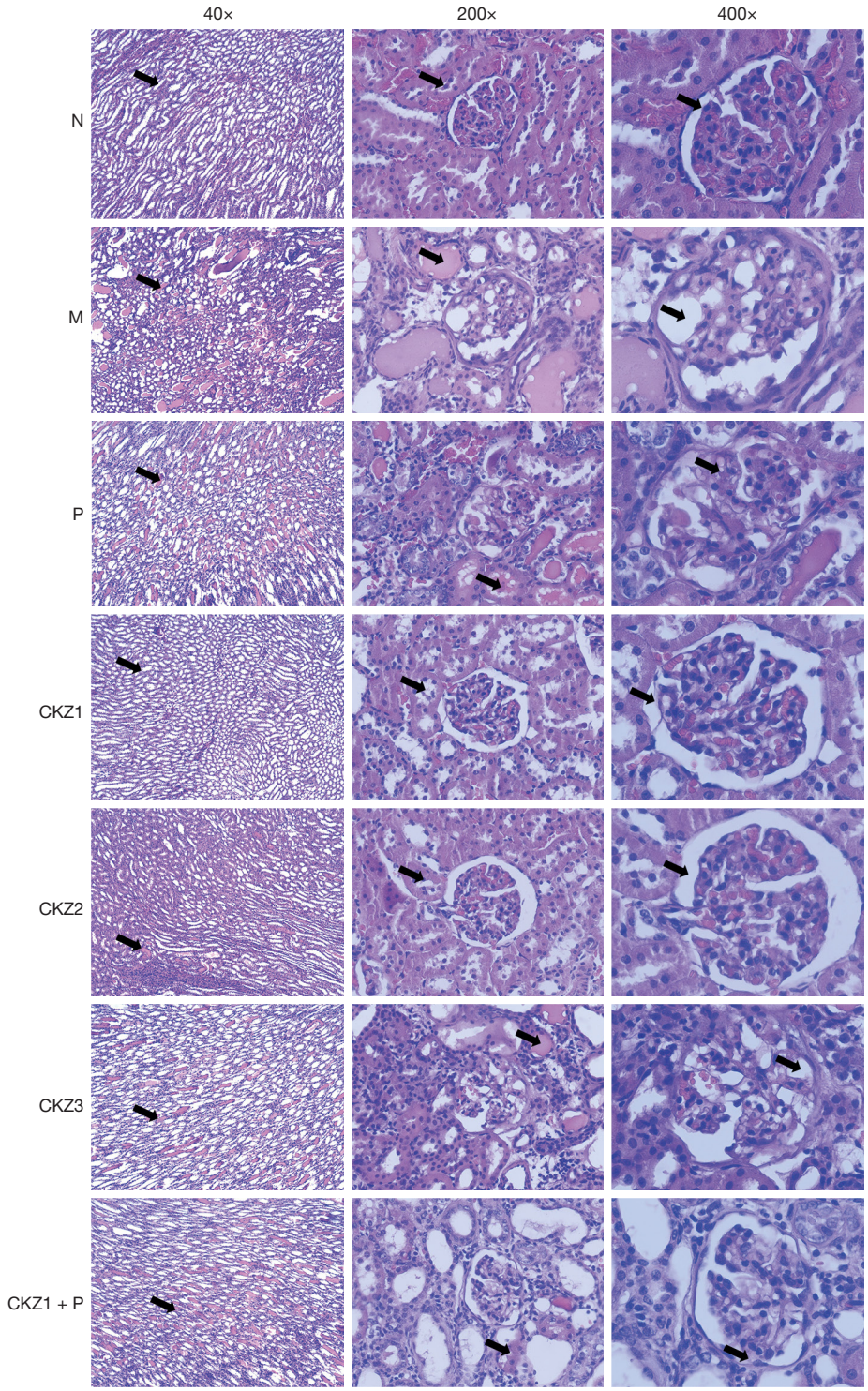
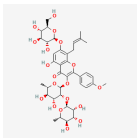
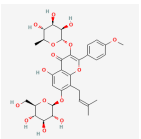
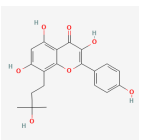
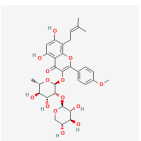
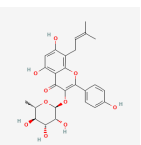
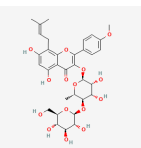
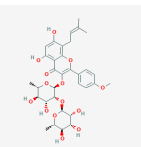
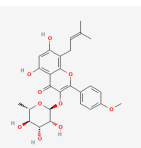
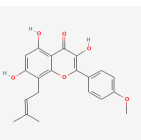
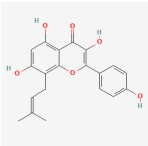
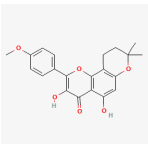


Figure 2 HE stained pathological sections of kidneys from rats in each group after intervention. Black arrows: the site of pathological changes in the collecting ducts, tubules or glomerulus. HE, hematoxylin and eosin; CKZ, Chuankezhi injection. N: the control group; M: the model group; P: the prednisone group; CKZ1: the high-dose CKZ group; CKZ2: the medium-dose CKZ group; CKZ3: the low-dose CKZ group; CKZ1+P: the high-dose CKZ + prednisone group.

Table 2 Metabolic components of CKZ

Component	Molecular formula	Structure
Epimedin C (CKZDX1)	$C_{39}H_{50}O_{19}$	
Icariin (CKZDX2)	$C_{33}H_{40}O_{15}$	
Noricaritin (CKZDX3)	$C_{20}H_{20}O_{7}$	
Sagittatoside B (CKZDX4)	$C_{32}H_{38}O_{14}$	
Baohuoside II (CKZDX5)	$C_{26}H_{28}O_{10}$	
Baohuoside VII (CKZDX6)	$C_{33}H_{40}O_{15}$	
2”-O-Rhamnosylicar-iside II (CKZDX7)	$C_{33}H_{40}O_{14}$	
Icariside II (CKZDX8)	$C_{27}H_{30}O_{10}$	
Icaritin (CKZDX9)	$C_{21}H_{20}O_{6}$	
Desmethyl Icaritin (CKZDX10)	$C_{20}H_{18}O_{6}$	
Beta-Anhydroicaritin (CKZDX11)	$C_{21}H_{20}O_{6}$	

CKZ, Chuankezhi injection.

components (*Table 2*), including (I) Epimedin C (CKZDX1), (II) Icariin (CKZDX2), (III) Noricaritin (CKZDX3), (IV) Sagitatoside-B (CKZDX4), (V) Baohuoside-II (CKZDX5), (VI) Baohuoside-VII (CKZDX6), (VII) 2”-O-Rhamnosylicariside II (CKZDX7), (VIII) Icariside-II (CKZDX8), (IX) Icaritin (CKZDX9), (X) Demethyl-Icariin (CKZDX10), and (XI) beta-Anhydroicaritin (CKZDX11). The corresponding targets of the 11 active components were identified through the SwissTargetPrediciton database, and the duplicates were combined and removed to obtain a total of 128 targets.

Compound-target network

A compound-target network was built to examine the interaction between CKZ active compounds and their potential targets. There were 140 nodes and 465 edges in this network, in which pink represented CKZ (1), green represented active components (11), and blue represented the corresponding targets of active components (128). Using the degree value ranking, we found that the key nodes were Icariside II, Icaritin, Desmethyl Icaritin, and beta-Anhydroicaritin (*Figure 3A*).

The therapeutic targets of CKZ for SR FSGS

Information of the targets was downloaded from the GeneCard and OMIM disease databases, combined, and consolidated to obtain a total of 479 disease targets for SR FSGS. Subsequently, 128 CKZ active ingredient targets

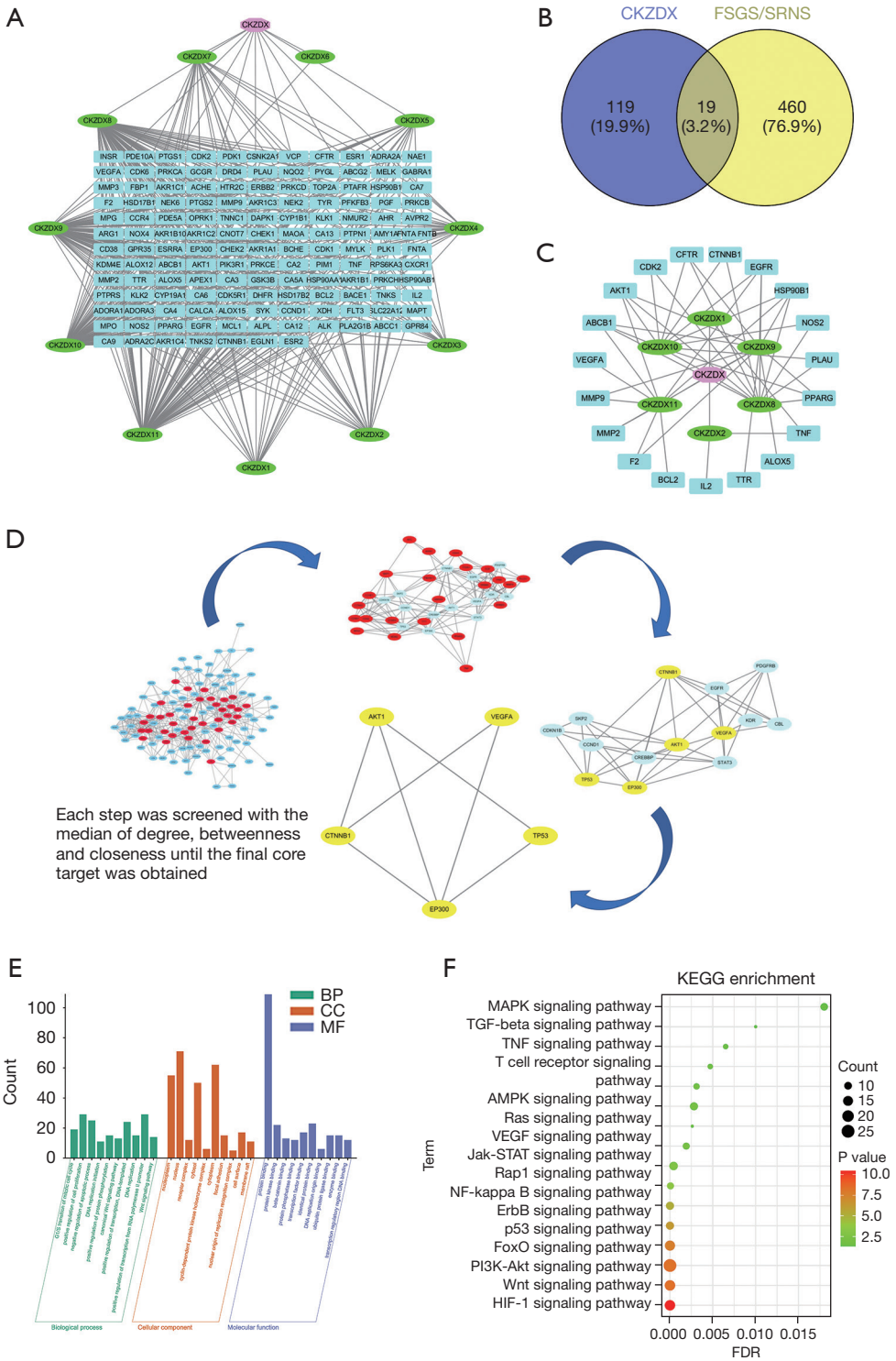


Figure 3 GO and KEGG pathway enrichment analysis and network model for network pharmacology. (A) Target of active CKZ compounds. (B) Therapeutic targets of CKZ. (C) CKZ-Active compounds-Therapeutic targets network. (D) PPI construction of therapeutic target of CKZ. (E) GO enrichment of key CKZ targets. (F) KEGG enrichment of key CKZ targets. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; BP, biological process; CC, cellular component; MF, molecular function; CKZ, Chuankezhi injection; FDR, false discovery rate.

were matched with 479 SR FSGS disease targets, and finally, 19 common targets of CKZ and SR FSGS were obtained (*Figure 3B*). By acting on these overlapping genes, CKZ may be useful in treating SR FSGS. A total of 19 common targets of CKZ for SR FSGS corresponded to 6 active components, including Epimedin C (CKZDX1), Icariin (CKZDX2), Icariside II (CKZDX8), Icaritin (CKZDX9), Desmethyl Icaritin (CKZDX10), and beta-Anhydroicaritin (CKZDX11). The network had 26 nodes and 49 edges, with pink representing CKZ (1), green representing active components (6), and blue representing therapeutic targets (19) (*Figure 3C*).

PPI network

To better understand how CKZ affects SR FSGS, we needed to clarify protein interaction and mutual interference. The STRING database's PPI data was used to create a PPI network with 19 potential therapeutic targets. This PPI network had 117 nodes and 512 edges. The 117 nodes were considered to be predicted therapeutic targets. It was assumed that all PPI network nodes that had DC, BC, and CC topological characteristics greater than the average value were considered hub nodes (critical targets) using network models and algorithms. Finally, VEGFA (degree =21), AKT1 (degree =24), TP53 (degree =28), EP300 (degree =29), and CTNNB1 (degree =34) were identified as key therapeutic targets (*Figure 3D*).

GO and KEGG analysis

We used DAVID 6.8 was utilized to carry out GO and KEGG enrichment analyses on 117 predicted therapeutic targets. The biological process, cellular component, and molecular function were examined separately, $P<0.05$ was set, and the top 10 enrichment results were considered. The results showed that the negative regulation of apoptosis and the positive regulation of protein phosphorylation was significantly enhanced in biological process; the nucleus and cytoplasm increased considerably in cellular component; and the protein binding and protein kinase binding was greatly enriched in molecular function (*Figure 3E*).

Following KEGG enrichment analysis, we discovered that CKZ had a significant impact on pathways such as PI3K-Akt signaling, HIF-1 signaling, Wnt signaling, FoxO signaling, p53 signaling, ErbB signaling, NF-kappa B signaling, Rap1 signaling, jak-STAT signaling, vascular endothelial growth factor (VEGF) signaling, and so on (*Figure 3F*).

Validation of molecular docking

We screened 6 active components from the therapeutic targets of CKZ for SR FSGS, including Epimedin C, Icariin, Icariside II, Icaritin, Desmethyl Icaritin, and beta-Anhydroicaritin. The docking structures for the 6 active compounds were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). ChemBio3D Ultra's energy minimizing function was used to obtain the lowest energy conformations, which were saved in MOL2 format and then imported into SYBYL-X.

The PPI screened out 5 critical therapeutic targets, including VEGFA, AKT1, TP53, EP300, and CTNNB1, which were then compared with the top 10 most enriched pathways and combined with the literature research. The target gene AKT1 was selected as the key therapeutic target for the binding validation experiment. The PDB data on AKT1 was used to identify the molecular structure of the receptor molecule (1UNQ), which was saved in MOL2 format and then imported into SYBYL-X.

We performed SYBYL-X to validate the accuracy of network pharmacology predictions by assessing the binding activity of 6 ligand and receptor molecules (1UNQ). The total score of Icariin, Icariside II, Epimedin C, Icariin, and Noricaritin with 1UNQ exceeded 5 points, which showed that the matching binding effect was perfect, among which Icariin showed the best matching binding effect, with a total score of 6.9329 (*Figure 4*).

Relative mRNA expression of PI3K and Akt in kidney tissues

To further investigate the relationship between the efficacy of CKZ on SR FSGS and the PI3K-Akt pathway, we examined the relative mRNA expression of PI3K and Akt in kidney tissues. Compared with the N group, the relative mRNA expression of PI3K and Akt in the M group significantly decreased ($P<0.001$). In contrast, CKZ increased the relative mRNA expression of PI3K and Akt in a dose-dependent manner. Besides, the CKZ1+P group could not increase the relative mRNA expression of PI3K and Akt in the SR FSGS rats (*Figure 5A,5B*).

Protein phosphorylation of PI3K and Akt in kidney tissues

To further investigate the relationship between the efficacy of CKZ on SR FSGS and the PI3K-Akt pathway, we examined the protein phosphorylation of PI3K and Akt in kidney

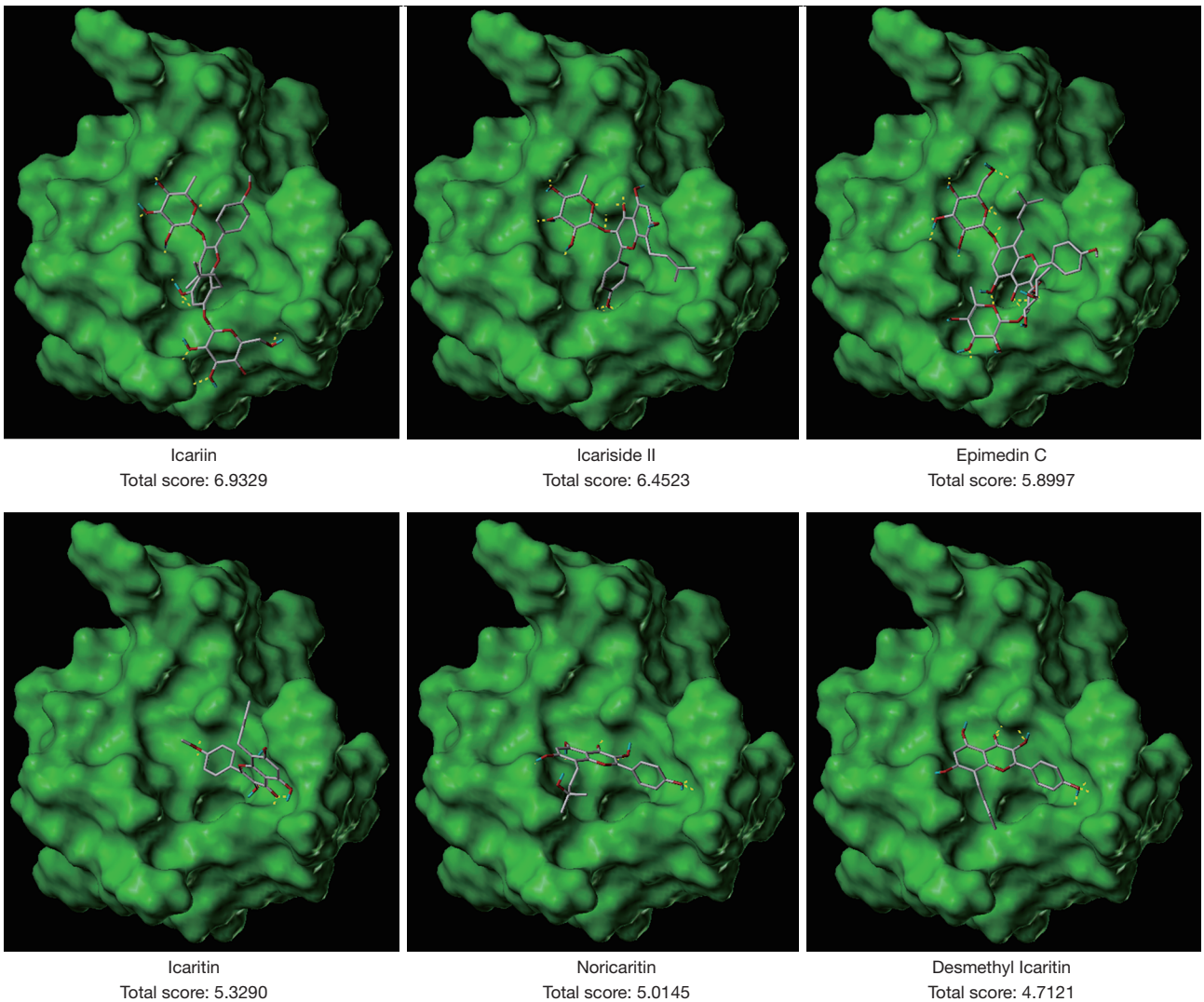


Figure 4 Molecular docking of AKT1 (1UNQ) with 6 active compounds of CKZ. CKZ, Chuankezhi injection.

tissues. Compared with the N group, the ratios of PI3K phosphorylation to total PI3K and Akt phosphorylation to total Akt in the M group significantly decreased (P<0.001). In contrast, CKZ increased the ratios of PI3K phosphorylation to total PI3K and Akt phosphorylation to total Akt in a dose-dependent manner. Besides, the CKZ1+P group could not increase the ratios of PI3K phosphorylation to total PI3K and Akt phosphorylation to total Akt in the SR FSGS rats (*Figure 6A-6C*).

Discussion

The results of this study showed that intramuscular injection of CKZ had a dose-dependent effect in SR FSGS model rats, including lowering urine protein, increasing serum ALB, lowering CHOL and TG, and treating pathological lesions in the kidney. Notably, the high-dose CKZ group and the control group showed no significant difference in ineffectiveness. These findings support our earlier hypothesis that SR FSGS might be a new indication of CKZ. The curative effect of CKZ on SR FSGS is significantly better than that of oral *Epimedium sagittatum* or *Morinda officinalis* reported in previous studies (8,9).

The main components of *Epimedium sagittatum* and *Morinda officinalis* are flavonoids, which are the main effective components of CKZ (24). The metabolic

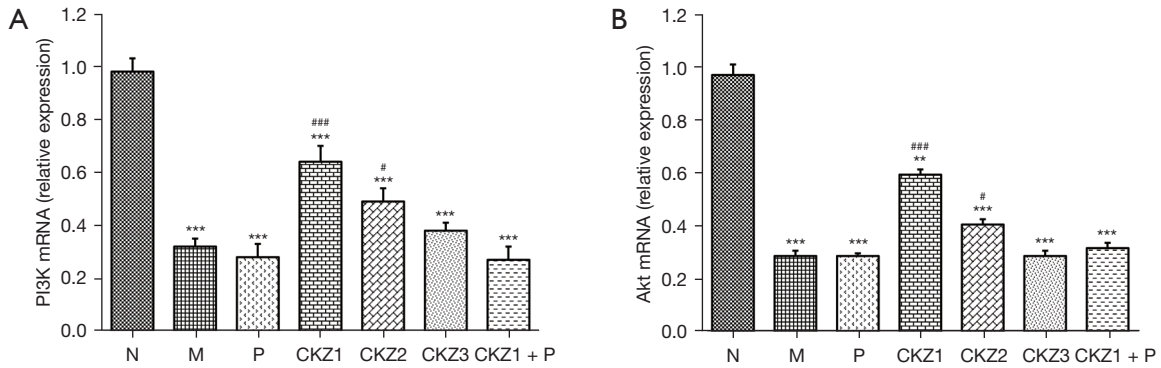


Figure 5 Relative mRNA expression of PI3K and Akt in kidney tissues. (A) Relative mRNA expression of PI3K in kidney tissues. (B) Relative mRNA expression of Akt in kidney tissues. N: the control group; M: the model group; P: the prednisone group; CKZ1: the high-dose CKZ group; CKZ2: the medium-dose CKZ group; CKZ3: the low-dose CKZ group; CKZ1+P: the high-dose CKZ + prednisone group. Data are means ± SEM (n=6). **, P<0.01, ***, P<0.001 *vs.* the N group; #, P<0.05, ###, P<0.001 *vs.* the M group, tested by one-way ANOVA. mRNA, messenger RNA; CKZ, Chuankezhi injection; SEM, standard error of the mean; ANOVA, analysis of variance.

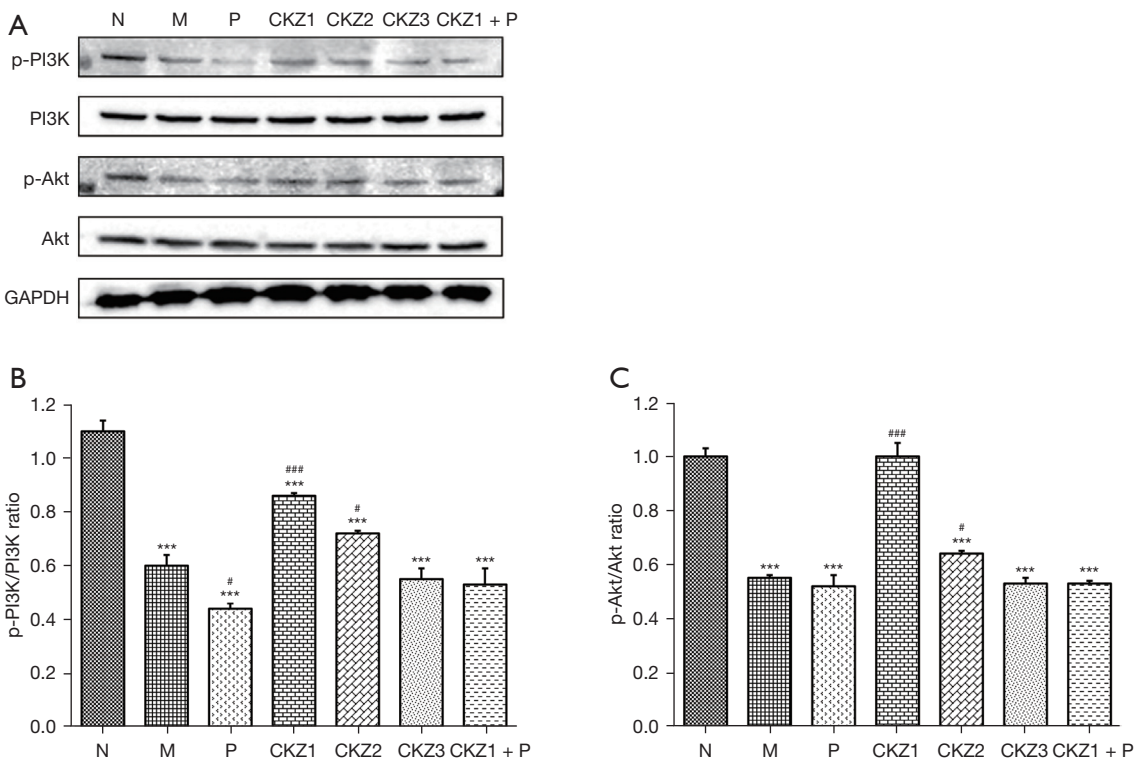


Figure 6 Protein phosphorylation of PI3K and Akt in kidney tissues. (A) Representative images of immunoblots of PI3K and Akt proteins in rat kidney tissues. (B) The ratio of PI3K phosphorylation to total PI3K in kidney tissues. (C) The ratio of Akt phosphorylation to total Akt in kidney tissues. N: the control group; M: the model group; P: the prednisone group; CKZ1: the high-dose CKZ group; CKZ2: the medium-dose CKZ group; CKZ3: the low-dose CKZ group; CKZ1 + P: the high-dose CKZ + prednisone group. Data are means ± SEM (n=3). ***, P<0.001 *vs.* the N group; #, P<0.05, ###, P<0.001 *vs.* the M group, tested by one-way ANOVA. CKZ, Chuankezhi injection; SEM, standard error of the mean; ANOVA, analysis of variance.

transformation pathway of flavonoids can occur before entering the blood, with the participation of gastrointestinal and intestinal flora, or after entering the blood (25). Before entering the blood, the flavonoids will experience a significant first-pass elimination in the liver by oral administration (26). Therefore, in previous studies (8,9), when oral administration of *Epimedium sagittatum* or *Morinda officinalis* was applied to treat FSGS, under the influence of impaired gastrointestinal function and intestinal flora imbalance, the body may not have been able to carry out normal drug metabolism and transformation. In addition, affected by the first clearance of the liver, the flavonoids entering the blood were decreased significantly and affected the further metabolic transformation after entering the blood. When using CKZ, intramuscular injection can avoid the above 2 situations, which may be the reason why CKZ can achieve a better curative effect compared with previous studies (8,9). In conclusion, according to this study, CKZ may be more effective than oral TCM in the treatment of SR FSGS, which may be associated with the different drug metabolism caused by the intramuscular administration of CKZ.

In this study, 6 active components were screened from the therapeutic targets of CKZ for FSGS: Epimedin C, Icariin, Icariside II, Icaritin, Desmethyl Icaritin, and beta-Anhydroicaritin; 5 key therapeutic targets screened out from PPI: including VEGFA, AKT1, TP53, EP300, and CTNNB1. The negative regulation of apoptosis and the positive regulation of protein phosphorylation was dramatically strengthened. Pathways such as PI3K-Akt signaling, HIF-1 signaling, Wnt signaling, FoxO signaling, and p53 signaling were significantly improved; 5 active components which may treat SR FSGS were well matched and combined with AKT1 in the molecular docking. Among the above-mentioned network pharmacological analysis, AKT1, protein phosphorylation, and PI3K-Akt signaling pathway were closely associated with SR FSGS. In mechanistic studies of SR FSGS, it was confirmed that podocyte injury is the critical factor in the pathogenesis, and the prevention and treatment of podocyte injury is the key to the treatment. At the same time, the PI3K-Akt signaling pathway was shown to be critical in investigations on podocytes; PI3K is one of the classical upstream substances regulating Akt. Its essence is a lipid second messenger, which is associated with intracellular signal transduction. It can activate Akt, phosphorylate Akt, and then exert downstream physiological effects (27). Studies have indicated that activation of the PI3K-Akt signaling pathway is an important way to reduce glomerular podocyte apoptosis and maintain the morphological and functional integrity of podocytes (28,29). Similar studies have also shown that activating Akt can protect podocytes in the process of chronic kidney disease (30,31). Therefore, we can predict that AKT1 is a key target gene, PI3K-Akt signaling is a critical pathway, and positive regulation of protein phosphorylation is an important biological process in SR FSGS. The qPCR and WB analyses revealed that CKZ dramatically increased the relative mRNA expression and protein phosphorylation of PI3K and Akt, which was consistent with the prediction in network pharmacological analysis. In conclusion, according to this part of the study, the active components in CKZ may help treat SR FSGS by activating PI3K-Akt signaling.

In this study, CKZ combined with prednisone did not alleviate or eliminate SR, but annulled the ameliorative effect of CKZ. This interesting phenomenon may be associated with the antagonistic effect of prednisone in a specific link of the mechanism of action of CKZ. Does prednisone play a negative role in a specific link of this pathway? Steroids have been shown to inhibit the phosphorylation of Akt (32) and the PI3K-Akt signaling pathway (33,34) in a variety of cells. In detecting the relative mRNA expression and protein phosphorylation of PI3K and Akt in rat kidney tissues, the results showed that the relative mRNA expression and protein phosphorylation of PI3K and Akt were significantly reduced in the CKZ combined with prednisone groups, compared with the CKZ group. In conclusion, according to this study, when CKZ was combined with prednisone, prednisone decreased the relative mRNA expression and protein phosphorylation of PI3K and Akt and antagonized the activation of PI3K-Akt signaling by CKZ, thereby nullifying the ameliorative effect of CKZ.

Network pharmacology is a new idea to study TCM. It can not only explain the mechanism of action of TCM but also provide direction for TCM to find new indications. However, there are also some problems worth thinking about:

(I) The chemical composition of TCM is not completely clear. At present, various databases used for network pharmacological analysis do not fully provide the chemical components of TCM. Therefore, the analysis based on incomplete chemical components may be incomplete, and even some important information may be omitted. (II) The network pharmacological analysis method does not take into account the possible impact of different components of

TCM on the analysis. At present, various methods used in network pharmacological analysis start with the components of TCM, but ignore the content of components. The dose-effect relationship is recognized as an important part of drug research. It is divorced from the role of content to analyze and predict components, which has loopholes. For example, there are 10 components of TCM displayed in the database. After network pharmacological analysis and prediction, it is found that one component may be the key component for the treatment of a disease, but in fact, the content of this component in the TCM is very low, so the prediction result is likely to be divorced from the actual situation, and the drug action mechanism further analyzed by this prediction result is likely to be wrong. (III) The current method of screening components from OB testing (35) in the Chinese medicine composition database used for network pharmacological analysis is not suitable for the analysis of non-oral TCM. Therefore, for the network pharmacological analysis of non-oral TCM, it is better to obtain the chemical components by mass spectrometry analysis and fingerprinting analysis first, and then continue the network pharmacological analysis. (IV) The current network pharmacological analysis basically starts from the original components of TCM, but there is a general rule that the original components of TCM are ineffective in *in vitro* experiments but effective in *in vivo* experiments, which indicates that in many cases, the effects of TCM occur through a complex metabolic transformation *in vivo*, rather than the direct effects of the original components. For example, in this study, the main component of CKZ, flavonoids, entered the body and very few original components could be detected, which were basically converted into other metabolic components (23), which are more likely to be the key to cause the effect. This suggests to us that it may be a better choice to choose the *in vivo* metabolic components of TCM for network pharmacological analysis.

In conclusion, when we use network pharmacology to study TCM, in addition to the classical method of using the original drug components for analysis, we can also try to use the *in vivo* metabolic components of TCM for analysis, and we can compare the results of both analyses, and we may find some interesting or meaningful findings. If network pharmacology is used to elucidate the mechanism of action of TCM, it is important to pay attention to the content of the components within TCM. If the predicted mechanism corresponds to a component that is very low in TCM, it is important to pay attention to the fact that such results are likely to be unreliable. If the predicted new indication corresponds to a component with very low content in the TCM, it is also important to pay attention to the fact that although this component is likely to be the key to treating the new indication, the TCM is actually unable to play a role in treating the new indication due to its very low content in the TCM. Finally, no matter which of the above is the case, it has to be verified by experiments, and theoretical analysis of network pharmacology alone is not enough.

This study explored the curative effect and mechanism of action of CKZ on SR FSGS. In conclusion, the valuable findings may provide a new method for treating SR FSGS. In the future, we will perform further studies on CKZ. We plan to use current mainstream drugs for treating SR FSGS as positive controls to more thoroughly evaluate the efficacy of CKZ. Also, we will use metabolomic methods to further explore the mechanism of action of CKZ. In addition, to improve patients' compliance, we plan to research the oral administration of CKZ to find more effective and convenient treatments for patients with SR FSGS.

Conclusions

This study verified the effect of CKZ (a TCM injection extracted from *Epimedium sagittatum* and *Morinda officinalis*) on SR FSGS and explored its mechanism of action. The study data showed that intramuscular injection of CKZ played a significant role, and the curative effect was very significant, which may be associated with the activation of PI3K-Akt signaling by CKZ. The treatment of SR FSGS may be a new indication of CKZ.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1962/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1962/dss

Conflicts of Interest: All authors have completed the ICMJE

uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1962/coif). LGG, YKL and YRZ are from Guangzhou ImVin Pharmaceutical Co., Ltd. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. TCMF1-2020019) granted by the animal care committee of The First Affiliated Hospital of Guangzhou University of Chinese Medicine, in compliance with the *Regulation on the Administration of Laboratory Animals* (2017 Revision) for the care and use of animals.

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