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Research article

Drug pair of Cornus officinalis and Radix achyranthis bidentatae improves renal injury of hypertension by regulating metabolic reprogramming mediated by eNOS

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ABSTRACT

Objective: To explore the effects and possible mechanisms of the drug pair Cornus officinalis and Radix achyranthis bidentatae (SYR-NX) on improving hypertensive kidney damage.
Method: SYR-NX, a formulation of Cornus officinalis and Radix Achyranthis Bidentatae with a dose ratio 1:2.5, was used in this experiment. We investigated the effects of SYR-NX on spontaneously hypertensive rats (SHR) fed with a high-salt diet and Human Kidney-2 (HK2) cells exposed to hypoxia. After 8 weeks of treatment with SYR-NX, blood pressure was tested, and β 2-Microglobulin(β2-MG), blood creatinine (S-cr), endothelial nitric oxide synthase (eNOS), nicotinamide adenine dinucleotide phosphate (NADPH), M2 pyruvate kinase (PKM2), adenosine triphosphate (ATP), pyruvate, lactate, connective tissue growth factor (CTGF) and tumor necrosis factor-α (TNF-α)were measured. HK2 cells pre-treated with SYR-NX were cultured in a three-gas hypoxic incubator chamber (5 % CO_2, 1 % O_2, 94 % N_2) for 12 h, and then eNOS, PKM2, NADPH, ATP, pyruvate, lactate, CTGF and TNF-α were assessed.
Results: SYR-NX significantly reduced SBP, DBP, β2-MG, S-cr, PKM2, pyruvate, lactate, CTGF and TNF-α, and increased eNOS, NADPH, and ATP.
Conclusion: SYR-NX can regulate metabolic reprogramming through eNOS and improves hypertensive kidney injury.

1. Introduction

Hypertension is a common cardiovascular disease in China, and kidney damage is one of the main target organ damages in

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hypertensive patients, seriously endangering their life and health [1,2]. The latest guidelines recommend drugs such as angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, SLGT2 inhibitors, and finerenone as therapeutic options for hypertensive kidney damage. These drugs have certain therapeutic effects, but some patients still experience poor clinical outcomes. The current treatment of hypertensive kidney damage should not only emphasize the renal protective effect brought by achieving blood pressure reduction standards, but also pay attention to the unique renal protective effect of drugs. Traditional Chinese medicine has its unique therapeutic effect in treating hypertensive kidney damage [3–5], but there are still shortcomings in explaining the specific mechanism. This study aims to explore the specific molecular mechanisms of traditional Chinese medicine in preventing and treating hypertensive renal injury.

Under the guidance of the theory of "blood stasis and heat" proposed by Professor Zhou Zhongying [6], a master of traditional Chinese medicine, our research group proposes that "liver and kidney yin deficiency, blood stasis obstructing kidney meridians" are the main pathogenesis of hypertensive kidney injury by conducting extensive clinical observation and summary analysis. Based on the characteristics of this pathogenesis, the treatment of hypertensive kidney injury using the methods of clearing the liver and tonifying the kidney, resolving blood stasis and unblocking collaterals can effectively reduce urinary protein [7,8]. SYR-NX, a formulation of Cornus officinalis and Radix achyranthis bidentatae with the dose ratio 1:2.5 has been widely used in clinical practice for many years. It is safe and effective in treating hypertensive kidney injury by clearing the liver and tonifying the kidney, resolving blood stasis and unblocking collaterals. The preliminary researches of the project team have shown that the methods of clearing the liver and tonifying the kidney, resolving blood stasis and unblocking collaterals can play a significant role in the treatment of hypertensive kidney injury, including antioxidant, anti-inflammatory, and anti fibrotic effects [9–15].

Hypertensive kidney damage is the second leading cause of death for chronic kidney disease (CKD) worldwide, increasing the risk of end-stage kidney disease, cardiovascular adverse events, and sudden death. Ischemia and hypoxia are important mechanisms of hypertensive kidney damage. In a diseased state, renal tubular epithelial cells undergo ischemia and hypoxia, leading to metabolic changes, increased glycolytic pathways, and imbalanced oxidation-reduction, thereby exacerbating inflammatory fibrosis and further exacerbating hypertensive renal damage. Among them, glucose metabolism reprogramming plays a crucial role. The changes in metabolic patterns are collectively referred to metabolic reprogramming, and their role in kidney diseases has received increasing attention in recent years. The kidney is a high-energy consuming organ, and a normal and orderly energy metabolism system is the biochemical basis for maintaining the specific structure and physiological function of the kidney. Related studies have shown that the main feature of cell activation during renal fibrosis is the transition of renal cell metabolism from oxidative phosphorylation to glycolysis [16–21]. Inhibiting renal cell glycolysis can significantly alleviate renal fibrosis, and glycolysis inhibitors can become a potential anti fibrosis strategy. Researchers [16] found that metabolic reprogramming based on eNOS has a protective effect on kidney injury in their study published in "Nature". ENOS can reduce PKM2, decrease glycolysis, decrease pyruvate, increase Pentose phosphate pathway (PPP) related intermediates, thereby promoting inflammation and fibrosis, and promoting kidney damage. However, no Western medicine has been found to improve metabolic reprogramming through this pathway for clinical treatment of hypertensive kidney damage.

On the basis of good preliminary research and literature review [9–15], we propose the following scientific hypothesis: SYR-NX may inhibit renal cell oxidation and inflammatory fibrosis by regulating the eNOS related pathway, upregulate eNOS activity, inhibit PKM2, reduce glycolysis, lower pyruvate, increase PPP related intermediates, thereby inhibiting inflammatory fibrosis, and thus improving hypertensive kidney damage.

2. Materials and methods

2.1. Preparation of drug pair of Cornus officinalis and Radix achyranthis bidentatae (SYR-NX)

SYR-NX was composed of 6 g Cornus officinalis and 15 g Radix achyranthis bidentaae, purchased from Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine. The raw medicines were prepared and mixed according to the dosage of its components. They were soaked in distilled water for 30 min, boiled twice, and the filtrates were taken and combined. The mixture was evaporated and concentrated in 80 °C water bath to contain 50 mg raw medicinal materials per 1 mL. The mixture was stored in a refrigerator at 4 °C, shaken well and heated to room temperature before use.

2.2. Animals and treatment

30 Spontaneously hypertensive rats (SHR) and 10 Wistar-Kyoto rats (WKY), 8 weeks old, male, weighing 230 ± 20 g. Provided by Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. Rats were adaptively fed for 4 weeks and modeled at 12 weeks of age. Feeding, bedding and feeding environment: bedding: Shukebeta brand SPF grade experimental animal pellet bedding, produced by Jiangsu Synergy Pharmaceutical Biotechnology Co., Ltd. Feed: 4 % sodium chloride high salt rat pellet feed, 0.4 % sodium chloride normal salt rat pellet feed, produced by Jiangsu Collaborative Medical Biotechnology Co., Ltd. Rats in each group were free to forage and drink water, exposed to light/dark for 12 h, at room temperature (22 ± 2) °C and relative humidity (45–60)%. They were raised in the Basic Pharmacology Laboratory Animal Room (SPF level) of Nanjing University of Affiliated Hospital of Nanjing University of Chinese Medicine.

According to previous experiment's experience [15], 30 12-week-old SHR were randomly divided into three groups: model group, SYR-NX group, and the valsartan group. 10 WKY were used as the control group. SHR were fed with high salt (4 % Nacl) diet for 8 weeks. SYR-NX group was gavaged by 200 mg/kg/d Cornus officinalis and Radix achyranthis bidentatae decoction. The dose ratio of

Cornus officinalis and Radix achyranthis bidentatae was 1:2.5 according to the pharmacology experimental methodology of traditional Chinese medicine based on the results of the previous study which has significant therapeutic effect [15]. Valsartan group: Valsartan suspension was administered with 17 mg/kg/d. After 8 weeks' treatment of SYR-NX or valsartan, we collected the blood from abdominal aorta performed kidney tissue sampling. Serum creatinine (S-cr) and urinary β2-microglobulin (β2-MG) were assessed. The changes of endothelial nitric oxide synthase (eNOS), nicotinamide adenine dinucleotide phosphate (NADPH), M2 pyruvate kinase (PKM2), adenosine triphosphate (ATP), pyruvate, lactate, connective tissue growth factor (CTGF) and tumor necrosis factor-α (TNF-α) were also assessed in vivo. The Experimental Animal Ethics Committee of Affiliated Hospital of Nanjing University of Chinese Medicine approved all animal experiments on August 8, 2019 (Animal Ethics No.: 2021 DW-07-02).

2.3. Preparation of drug containing serum

20 Sprague Dawley rats (SD) were divided into the blank group (10 rats), SYR-NX group (5 rats) and valsartan group (5 rats). SYR-NX group was gavaged by 200 mg/kg/d SYR-NX. Valsartan suspension was administered with 17 mg/kg/d with a period of 7 days. Blood was collected from the abdominal aorta and centrifuged for 10 min at 3000 rpm [7]. The serum was inactivated at 56 °C for 30 min, packaged, and stored at −80 °C for future use.

2.4. Cells

Human renal tubular epithelial cells (HK2) were purchased from ATCC in the United States. Based on our previous experience in cell experiments [15], we cultured HK2 cells in a hypoxic incubator (5 % CO_2, 1 % O_2 and 94 % N_2) for 12 h to create the vitro model. We divided the cells into the blank group, model group, SYR-NX group, and valsartan group. Model group: hypoxia+10 % blank serum, SYR-NX group: hypoxia+5 % SYR-NX containing serum+5 % blank serum, valsartan group: hypoxia+10 % valsartan containing serum, control group: 10 % blank serum. The HK2 cells of the drug intervention group were pretreated with SYR-NX or valsartan containing serum for 1 h before modeling, and cultured in an hypoxic incubator for 12 h. The control group was cultured in a normoxic incubator until the end of the study. ENOS, NADPH, PKM2, ATP, pyruvate, lactate, CTGF, and TNF-α were observed.

2.5. Real-time quantitative PCR analysis

The total RNA was extracted by using Trizol (Ambion, USA). The High Capacity cDNA Reverse Transcription Kit (Vazyme, Nanjing, China) was used to conduct the reverse transcription. SYBR Green chemistry (Vazyme, Nanjing, China) on a 7500 fast RT-PCR system was used to perform amplified reaction. The primers (Invitrogen Co, Shanghai, China) were listed in Table 1. The ratio of the mRNA expression of the target gene vs that of β-actin was defined as $2^{-\triangle\triangle Ct}$.

2.6. Kits for ATP, lactate, pyruvate, eNOS, NADPH, PKM2, ATP, CTGF, and TNF- α

Levels of ATP, lactate, pyruvate, eNOS, NADPH, PKM2, ATP, CTGF, and TNF- α were measured using assay kits according to the instruction of manufacturer.

2.7. HE staining

Kidney tissues were fixed with 4 % paraformaldehyde for 24 h, dehydrated gradiently in turn, embedded in the wax block. Sticking the corresponding label of the rats. Wax block was sliced, dewaxed and washed. Hematoxylin staining and eosin staining were used in sequence. The samples were observed by microscope and the images were analyzed.

2.8. Masson staining

The kidney tissues were fixed and sectioned as HE staining. Weigert's iron hematoxylin staining to stain nucleus, ponceau S was used then for 5–10 min. After rinsing with distilled water, they were treated with molybdophosphoric acid treatment for 3–5 min. Samples were stained by Aniline blue again for 5 min and treated with 1 % glacial acetic acid for 1 min. Dry mount. The samples were observed by microscope and the images were analyzed.

Table 1
Primers used in Real-Time Quantitative PCR analysis.

Target Gene	Forward Primer	Reverse Primer
Human PKM2	5′-ATCCACGCTGGATAACGCCTAC-3′	5′-TGCCTTGCGGATGAATGACG-3′
Human TNF-α	5′-AGCCCATGTTGTAGCAAACC-3′	5′-TGAGGTACAGGCCCTCTGAT-3′
Human CTGF	5′-CTGCACCAGCATGAAGACAT-3′	5′-CTCCGGGACAGTTGTAATGG-3′
Human eNOS	5′-TCC TCA CCG CCT TCT CCC-3′	5′-CCT CAG GAT GTC CTG CAC GT-3′
Human β-actin	5′-GACCTGACTGACTACCTC-3′	5′-TCTTCATTGTGCTGGGTGC-3′

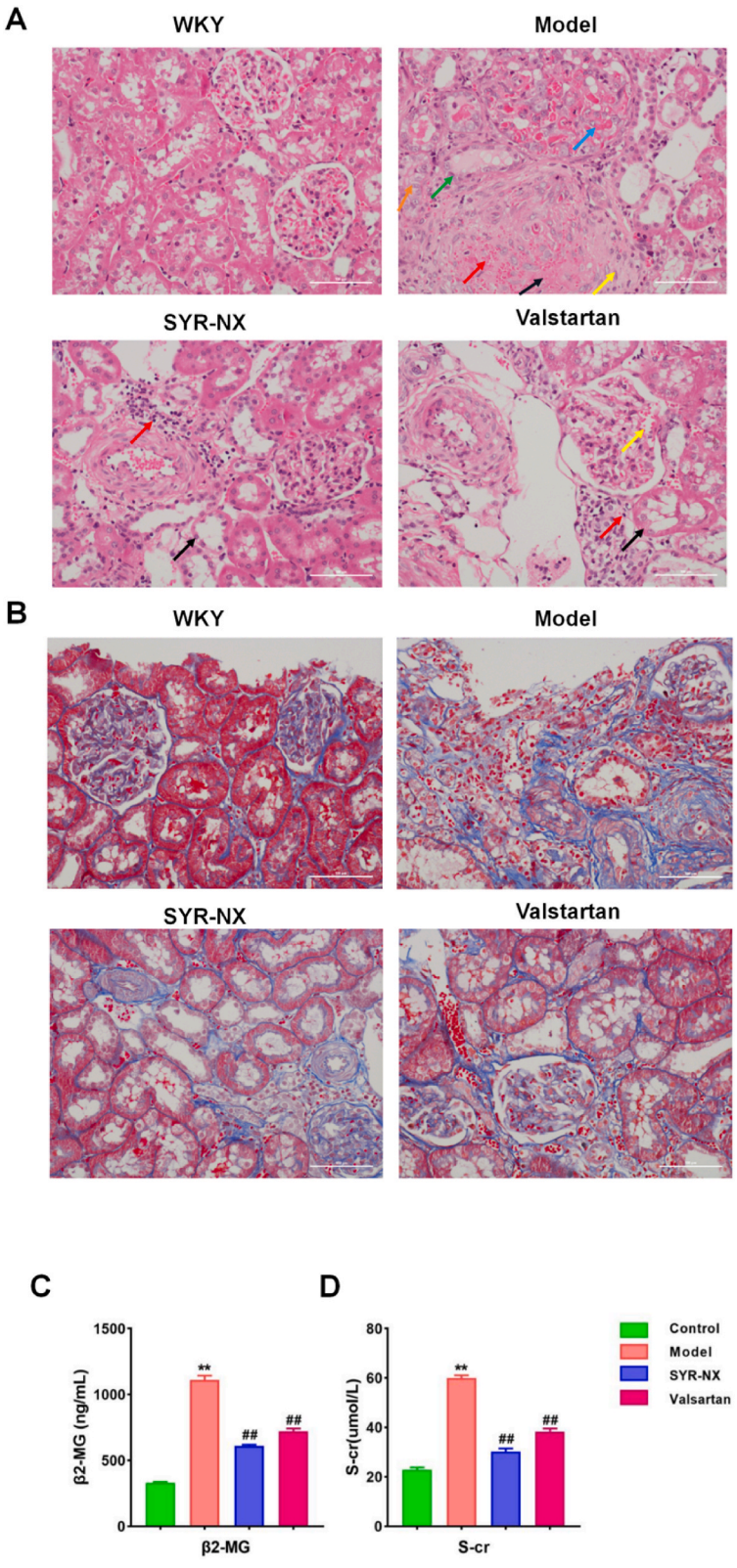

Fig. 1. SYR-NX promoted pathology, fibrosis and renal function in vivo. (A) Effects of SYR-NX on renal histomorphology in rats. (B) Effects of SYR-NX on masson staining method for staining renal fibrosis. (C) Effects of SYR-NX on β2-MG of high salt fed SHR. (D) Effects of SYR-NX on S-cr of high salt fed SHR.$^{**}P < 0.01$ vs WKY, $^{\#\#}P < 0.01$ vs model group.

2.9. Bioinformatics analysis and network pharmacology of SYR-NX

We retrieved the TCMSP database and screened for effective active ingredients based on oral bioavailability (OB $\geq$ 30 %) and drug like properties (DL $\geq$ 0.18). Then, we predicted the target compounds of candidate compounds of SYR-NX through the TCMSP database. We Collected target genes related to hypertensive kidney disease through GeneCards, OMIM, and NCBI Gene databases. We compared drug target genes with disease target genes using R software, screened common target genes between the then, and obtained intersection genes. We uploaded the intersecting target genes to the STRING platform and established a target protein interaction network (PPI). The protein interaction file string interaction (tsv) of common targets was obtained by exporting the STRING data platform. We imported it into Cytoscape 3.7.1 software and used the "CytoNCA" function to analyze its topological properties, screened out targets with a Degree greater than the average, and obtained the core targets. GO annotation and enrichment, KEGG annotation and enrichment were performed on the intersecting target genes. We determined relevant entries through KEGG signaling pathway enrichment analysis.

2.10. Identification of chemical components in SYR-NX based on Q-Orbitrap high-resolution liquid chromatography-mass spectrometry

Take 200 μL of the medicine solution, add 1000 μL 80 % methanol, and vortex for 10 min. Centrifuge at 4 °C for 10 min with a centrifugal force of 20000×*g*. Filter the upper clear liquid and analyze it on the machine. The data collected from high-resolution liquid quality analysis was completed through CD3.3 (Thermo Fisher), and after preliminary organization, database retrieval and comparison (mzCloud) were performed. Perform molecular docking using PyMOL2.3.2 3D molecular model design software.

2.11. Statistical analysis

SPSS 22 software was used to statistical analysis. All data conform to normal distribution and have equal variance and are expressed as mean ± standard deviation. Multiple group comparisons are conducted using one-way ANOVA, and pairwise comparisons between groups are conducted using Tukey's test. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. The effect of SYR-NX on pathology and fibrosis in high salt fed SHR

Effect of SYR-NX on renal histomorphology in rats was shown in Fig. 1A. In WKY group, the structure of renal glomeruli and tubules in the renal tissue are clear, the number of cells and matrix in the glomeruli are uniform, and the arrangement of renal tubular epithelial cells is regular. In model group, focal necrosis with disordered structures and eosinophilic homogeneous shapes can be seen (black arrow), accompanied by mild bleeding (red arrow). There is a small amount of connective tissue proliferation at the edge of the necrotic lesion (yellow arrow). A small amount of renal tubules are dilated, the lumen is enlarged, and the epithelial cells are flattened. Eosinophilic tissue fluid can be seen in the lumen (green arrow). A small amount of renal tubular epithelial cells are edematous, and the cytoplasm is loose and light stained (orange arrow). Capillary congestion and dilation can be seen in the glomeruli, and eosinophilic masses can be seen in some capillaries (blue arrow). In SYR-NX group, the structures of the glomerulus and renal tubules are clear, with a small amount of tubular dilation, enlarged lumen, flattened epithelial cells (black arrow). A small amount of inflammatory cell infiltration can be seen around blood vessels (red arrow). In valsartan group, the structures of the glomerulus and renal tubules are clear, with a small amount of renal tubular dilation and enlargement of the lumen (black arrow). There is a small amount of inflammatory cell infiltration around the blood vessels (red arrow). Occasional glomerular capillary dilation is observed (yellow arrow). After observing the effects of SYR-NX on masson staining method for staining renal fibrosis, it was found SYR-NX and valsartan effectively improved the renal fibrosis(Fig. 1B). The measurement results are shown in Table 2.

3.2. The effect of SYR-NX on BP, β2-MG and S-cr in high salt fed SHR

The effect of SYR-NX on the systolic blood pressure (SBP) of high salt fed SHR is shown in Table 3. When no drug intervention was implemented, SBP of SHR fed with high salt significantly increased compared to WKY ($P < 0.001$). After 4 weeks of administration, the SBP of the model group rats was higher than that of WKY group ($P < 0.001$). Compared with the model group, the SBP of SYR-NX and valsartan group significantly reduced ($P < 0.001$). After 8 weeks of drug intervention, the SBP of model group was significantly higher than that of WKY group ($P < 0.001$). Compared with the model group, the SBP of SYR-NX and valsartan group further decreased ($P <$

Table 2
The measurement results of masson staining.

Group	Positive pixel area	Total area of tissue pixel	Positive area proportion (%)
WKY	44806	1114719	4.02
Model	151129	1115121	13.55
SYR-NX	350075	4972800	7.04
Valsartan	105312	1228800	8.57

Table 3
Effect of SYR-NX on systolic blood pressure (SBP) of high salt fed SHR (Data showing means ± S.E.M, mmHg).

Group	Number (n)	before medication	(n)	after 4-week medication	(n)	after 8-week medication
WKY	10	126.10 ± 5.30	10	126.90 ± 5.36	10	126.70 ± 6.11
Model	10	185.60 ± 6.04[a]	8	186.88 ± 6.27[a]	8	183.88 ± 3.40[a]
SYR-NX	10	185.20 ± 7.42[a]	10	164.30 ± 7.53[b]	10	145.80 ± 4.29[b]
Valsartan	10	183.00 ± 8.46[a]	10	149.70 ± 6.70[b]	9	133.78 ± 5.17[b]

Note.
[a] $P < 0.01$ vs WKY.
[b] $P < 0.01$ vs Model.

0.001). The effect of SYR-NX on the diastolic blood pressure (DBP) of high salt fed SHR is shown in Table 4. When no drug intervention was implemented, SHR fed with high salt showed a significant increase in DBP compared to WKY ($P < 0.001$). After 4 weeks of intervention, the DBP of model group was significantly higher than that of WKY group ($P < 0.001$). Compared with model group, the DBP of SYR-NX and valsartan group significantly reduced ($P < 0.001$). After 8 weeks of drug intervention, compared with WKY group, the DBP of model group significantly increased ($P < 0.001$). Compared with model group, the DBP of SYR-NX and the valsartan group further decreased ($P < 0.001$). The levels of Blood S-cr and urine β2-MG of model group were significantly higher than that of WKY ($P < 0.001$). Compared with the model group, the levels of S–Cr and β2-MG of SYR-NX and valsartan group significantly decreased ($P < 0.001$). The results are shown in Fig. 1C and D.

3.3. Network pharmacological study on the treatment of hypertensive kidney disease with SYR-NX

We conducted network pharmacology studies on SYR-NX to identify its candidate active compounds and corresponding pathways for the progression of hypertensive nephropathy. Firstly, based on online databases and literature mining, an active compound library of two traditional Chinese medicines was constructed. Only compounds with good pharmacokinetic parameters, including oral bioavailability (OB ≥ 30 %) and drug similarity (DL ≥ 0.18) are included (Fig. 2C). Secondly, in order to elucidate the multiple interactions between target proteins, we constructed a drug compound target network (Fig. 2E). In order to understand possible regulatory mechanisms and their relationships, a PPI network was established (Fig. 2B). In order to further investigate the effect of SYR-NX on hypertensive nephropathy, 8716 hypertensive nephropathy related genes were extracted from the database, of which 111 genes overlapped with the potential target protein of SYR-NX (Fig. 2A). KEGG analysis of 111 gene targets suggests their involvement in the eNOS signaling pathway (Fig. 2D). Network pharmacology suggests that SYR-NX treatment for hypertensive kidney damage is closely related to eNOS.

3.4. Identification of chemical components in SYR-NX based on Q-Orbitrap high-resolution liquid chromatography-mass spectrometry

Q-Orbitrap high-resolution liquid chromatography-mass spectrometry was used to identify the chemical components of SYR-NX. The total ion flow diagram for chemical component identification is shown in Fig. 3A, and the main chemical components of SYR-NX are Asiatic acid, Gallic acid, and Oleanolic acid, as shown in Fig. 3B–D. In order to investigate whether chemical components participate in the regulation of e-NOS activation through direct interactions, we used Pymol 2.3.2 three-dimensional molecular model design software to predict the binding mode of the main components of SYR-NX with e-NOS (Fig. 3E–G). The results indicate that these compounds have binding sites with e-NOS, which may affect the activity or stability of e-NOS.

3.5. The effect of SYR-NX on eNOS, NADPH, PKM2, ATP, pyruvate, lactate, CTGF, TNF-α in high salt fed SHR

The levels of PKM2, pyruvate, lactate, CTGF, TNF-α in model group were significantly higher than those of the control group (PKM2: P=0.001, pyruvate: P=0.002, lactate, TNF-α: $P < 0.001$, CTGF: $P = 0.003$). The levels of eNOS, NADPH and ATP in the model group rats were significantly lower than those in the control group ($P < 0.001$). Compared with the model group rats, the levels of PKM2, pyruvate, lactate, CTGF, TNF-α in SYR-NX group significantly decreased (PKM2: P=0.008, pyruvate: $P < 0.001$, lactate: P=0.015, CTGF: P=0.025, TNF-α: $P = 0.001$). The levels of eNOS, NADPH and ATP in the group treated with SYR-NX were significantly

Table 4
Effect of SYR-NX on diastolic pressure (DBP) of high salt fed SHR (Data showing means ± S.E.M, mmHg).

Group	Number (n)	before medication	(n)	after 4-week medication	(n)	after 8-week medication
WKY	10	76.30 ± 6.22	10	78.10 ± 6.14	10	76.60 ± 7.81
Model	10	134.90 ± 7.88[a]	8	138.38 ± 6.26[a]	8	141.00 ± 5.15[a]
SYR-NX	10	137.10 ± 7.71[a]	10	129.90 ± 5.82[b]	10	108.50 ± 7.49[b]
Valsartan	10	137.60 ± 4.27[a]	10	97.80 ± 7.41[b]	9	89.33 ± 5.05[b]

Note.
[a] $P < 0.01$ vs WKY.
[b] P < 0.01 vs Model.

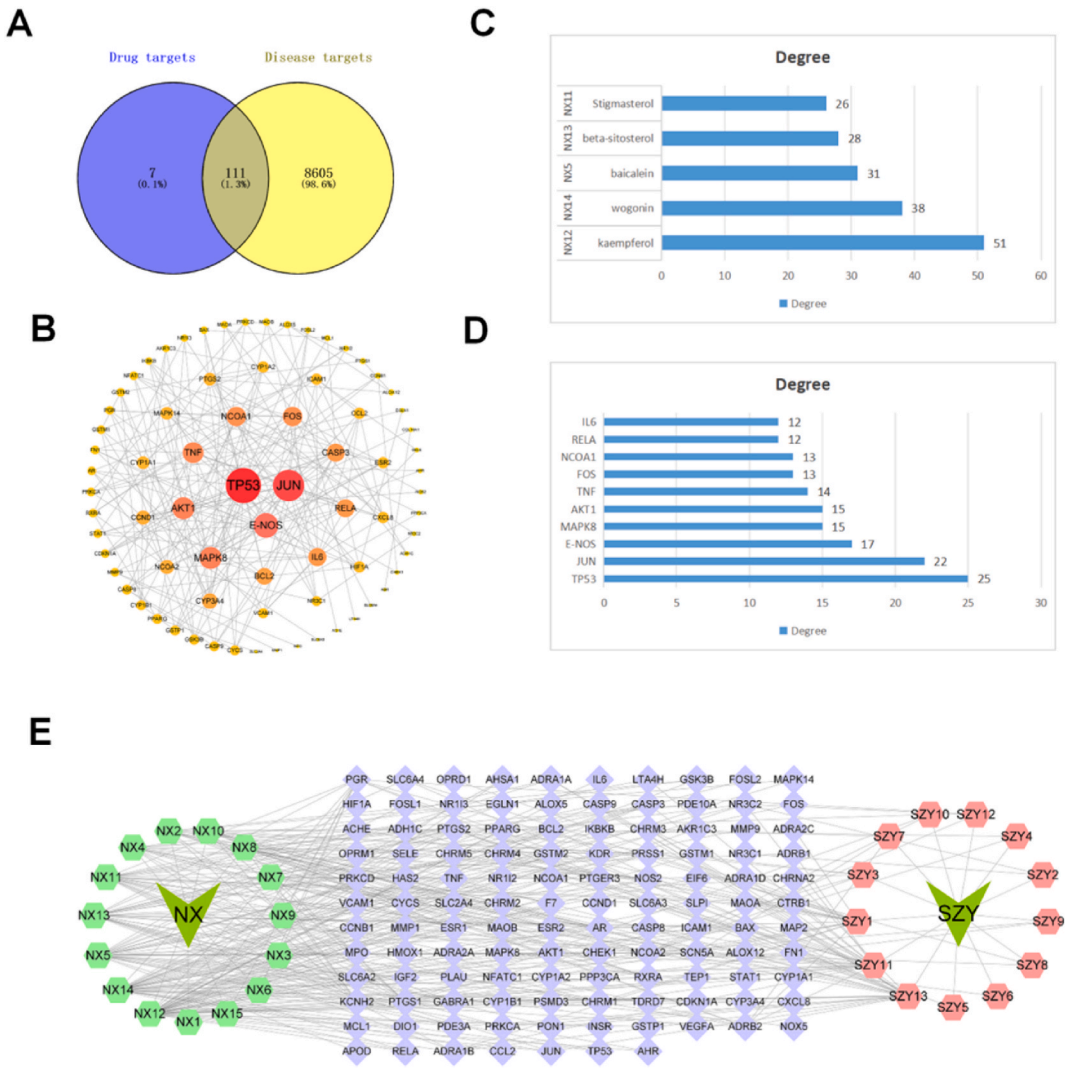

Fig. 2. Network pharmacology prediction of the potential active compounds in SYR-NX and corresponding pathways related to hypertensive nephropathy. (A) Figure of Venny. (B) Protein-protein interaction network. (C) List of core components. (D) Core Target list. (E) Figure of drug ingredient target.

higher than those in the model group ($P < 0.001$). The levels of pyruvate, CTGF and TNF-α in the valsartan group rats were significantly lower than those in the model group (pyruvate: $P < 0.001$, CTGF: P=0.038, TNF-α: $P = 0.026$). The eNOS, NADPH and ATP levels in the valsartan group rats were significantly higher than those in the model group ($P < 0.001$). The results are shown in Fig. 4.

3.6. *The effect of SYR-NX on eNOS, NADPH, PKM2, ATP, pyruvate, lactate, CTGF, TNF-α in hypoxia induced HK2 cells*

Firstly, the levels of eNOS, NADPH, PKM2, ATP, pyruvate, lactate, CTGF, TNF-α in hypoxia induced HK2 cells were detected by using kits. The levels of PKM2, pyruvate, lactate, CTGF, TNF-α in model group cells were significantly higher than those of the control group cells (PKM2: $P = 0.002$, pyruvate: $P < 0.001$, lactate, CTGF, TNF-α: $P < 0.001$). The levels of eNOS, NADPH and ATP in the model group cells were significantly lower than those in the control group (eNOS, NADPH: $P < 0.001$, APT: P=0.001). Compared with the model group, the levels of PKM2, pyruvate, lactate, CTGF, TNF-α of SYR-NX group cells significantly decreased (PKM2: $P = 0.005$, pyruvate: P=0.001, lactate: $P < 0.001$, CTGF: P=0.001, TNF-α: $P = 0.003$). The eNOS, NADPH and ATP levels in the group treated with SYR-NX were significantly higher than those in the model group (eNOS, NADPH: $P < 0.001$, ATP: $P = 0.024$). The levels of PKM2, pyruvate, lactate, CTGF and TNF-α in valsartan group cells were significantly lower than that of the model group (PKM2: $P = 0.032$, pyruvate: $P = 0.001$, lactate: $P = 0.001$, CTGF: P=0.002, TNF-α: $P = 0.006$). The levels of eNOS, NADPH and ATP in the valsartan group were significantly higher than that in the model group (eNOS, NADPH: $P < 0.001$, ATP: $P = 0.047$). The results are shown in Fig. 5. In addition, effects of SYR-NX on mRNA levels of eNOS, PKM2, CTGF and TNF-α in hypoxia induced HK2 cells were examined by using qPCR. The levels of PKM2, CTGF, TNF-α in model group cells were significantly higher than those of the control group cells

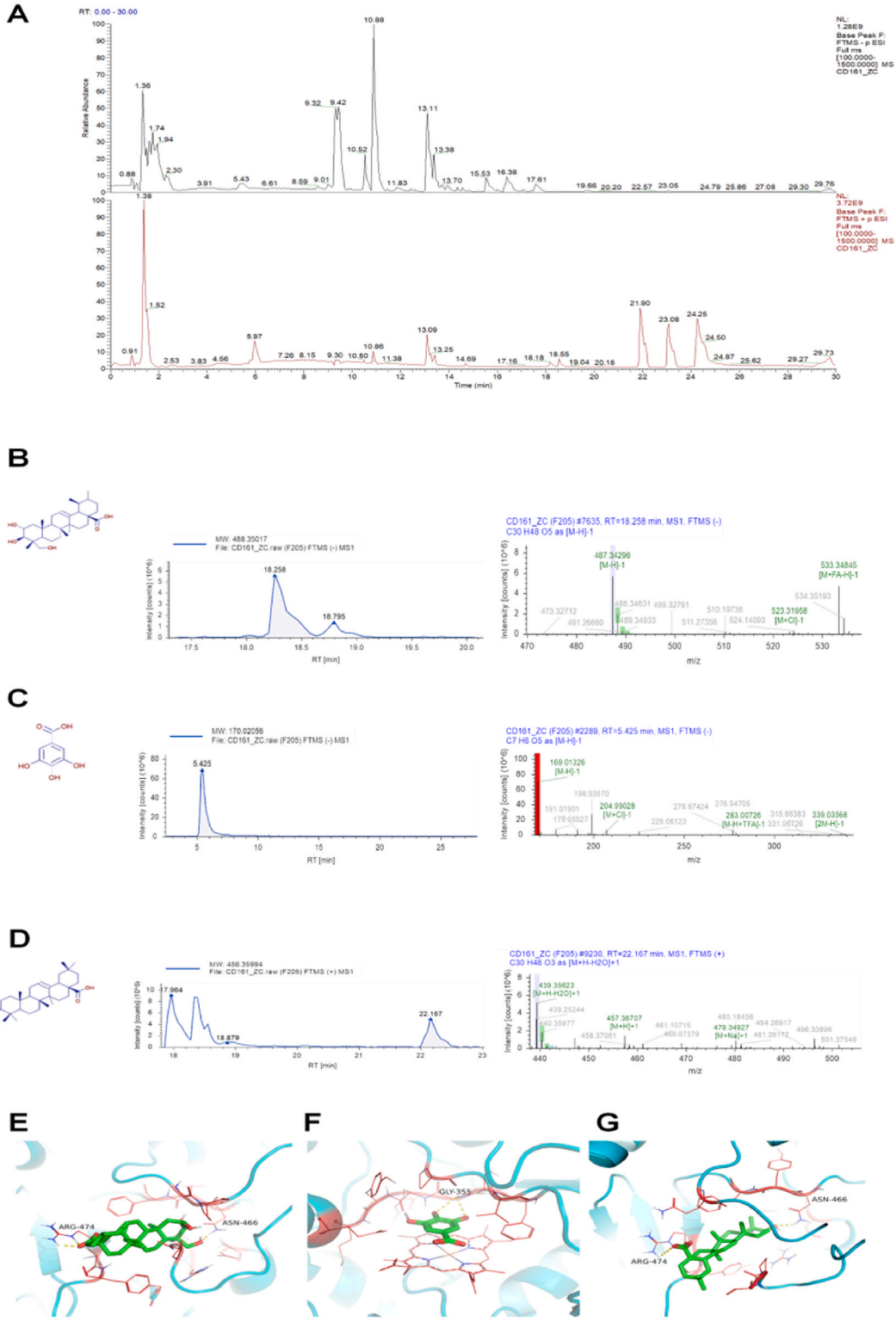

Fig. 3. Chemical composition of SYR-NX and analysis of the binding sites of the SYR-NX to e-NOS (A) Mass spectrometry analysis of SYR-NX. Chromatogram, and MS/MS image (B: Asiatic acid; C: Gallic-acid; D: Oleanolic acid). Possible binding sites to e-NOS (E: Asiatic acid; F: Gallic-acid; G:Oleanolic acid).

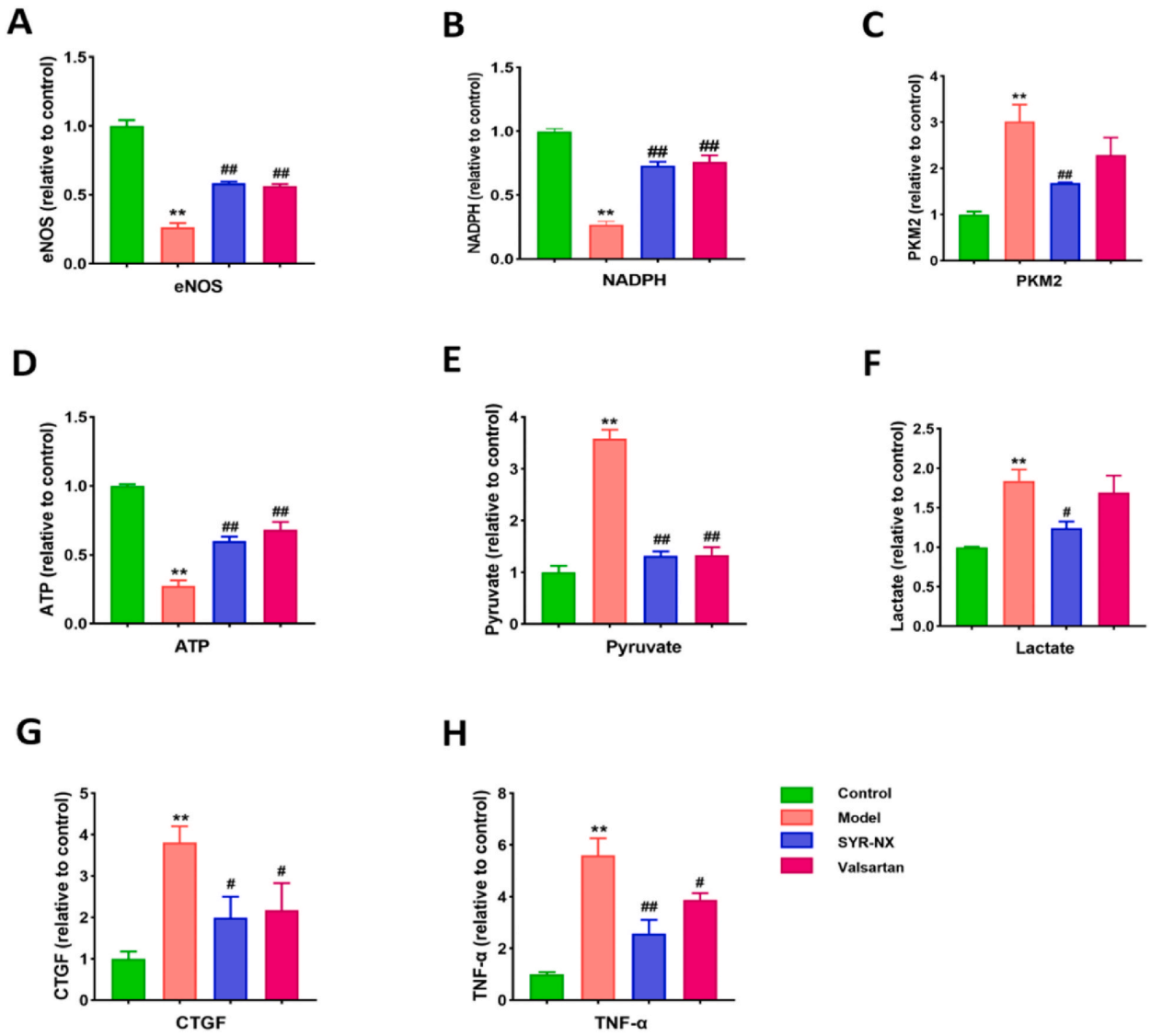

Fig. 4. Effect of SYR-NX on eNOS (A), NADPH (B), PKM2 (C), ATP (D), pyruvate (E), lactate (F), CTGF (G), TNF-α (H) of high salt fed SHR by using kits. $^{**}P < 0.01$ vs WKY, $^{\#}P < 0.05$ $^{\#\#}P < 0.01$ vs model group.

(PKM2: $P = 0.004$, CTGF: $P = 0.008$, TNF-α: $P = 0.013$). The levels of eNOS in the model group cells were significantly lower than those in the control group (eNOS: $P = 0.008$). Compared with the model group, the levels of PKM2, CTGF, TNF-α of SYR-NX group cells significantly decreased (PKM2: $P = 0.021$, CTGF: P=0.013, TNF-α: $P = 0.018$). The eNOS levels in the group treated with SYR-NX were significantly higher than those in the model group (eNOS: $P = 0.037$). The levels of PKM2, CTGF and TNF-α in valsartan group cells were significantly lower than that of the model group (PKM2: $P = 0.036$, CTGF: P=0.012, TNF-α: $P = 0.026$). The levels of eNOS in the valsartan group were significantly higher than that in the model group (eNOS: $P = 0.045$). The results are shown in Fig. 6.

4. Disscussion

Hypertensive kidney damage is one of the common diseases that damage human health and glucose metabolism reprogramming and inflammatory fibrosis play a key role in it. Metabolic reprogramming refers to changes in the metabolic mode of cells. In normal cells, energy is mainly obtained through oxidative phosphorylation, while in diseased states, energy is obtained through pathways such as glycolysis and pentose phosphate. The switching of energy acquisition modes by cells is called metabolic reprogramming [17]. Multiple studies [17–19] have shown that metabolic reprogramming plays an important role in the development of kidney disease. Yin [19] conducted vitro and vivo experimental verification of the relationship between glycolysis and renal fibrosis, and found that as the degree of renal fibrosis increased, the sugar metabolism enzymes in renal tissue increased synchronously. In vitro experiments, transforming growth factor-β1 (TGF-β1) can induce fibrosis of renal fibroblasts and undergo metabolic reprogramming, increase the

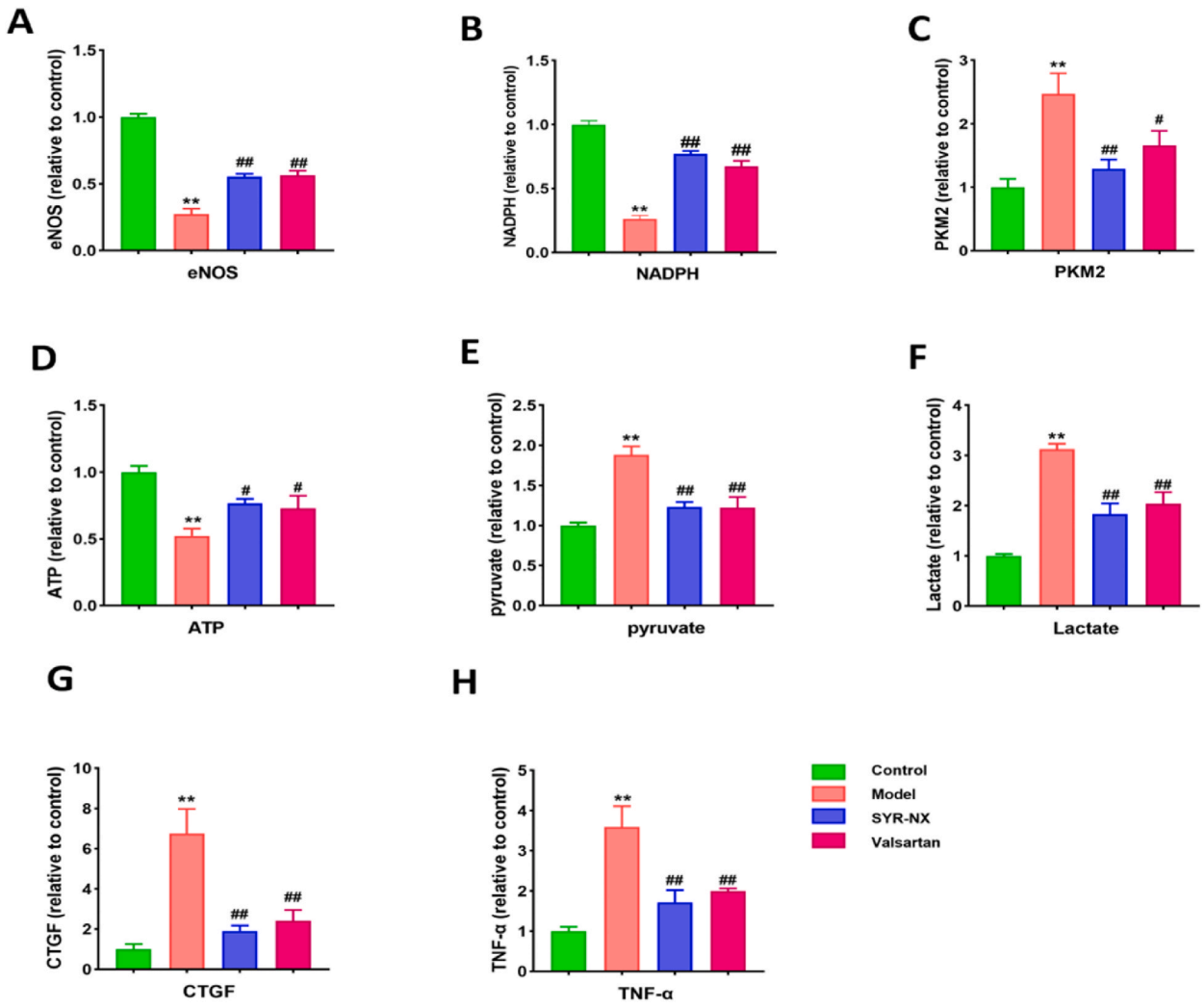

Fig. 5. Effect of SYR-NX on eNOS (A), NADPH (B), PKM2 (C), ATP (D), pyruvate (E), lactate (F), CTGF (G), TNF-α (H) of Hypoxia induced HK2 cells examined by using kits. $^{**}P < 0.01$ vs WKY, $^{\#}P < 0.05$ $^{\#\#}P < 0.01$ vs model group.

glycolytic pathway. Related studies [23] have shown that the transformation of renal cell metabolism from oxidative phosphorylation to glycolysis is the main feature of cell activation during renal fibrosis, and inhibiting renal cell glycolysis can significantly reduce renal fibrosis. Glycolysis inhibitors can serve as a potential anti fibrotic strategy for treatment.

The latest study published in the journal Nature [16] found that metabolic reprogramming regulated by the eNOS related pathway plays an important regulatory role in renal injury. From the perspective of metabolic reprogramming, this study elucidates the important role of eNOS in renal injury, providing us with new ideas for further prevention and treatment of hypertensive kidney injury from the perspective of metabolic reprogramming. Multiple studies [24–34] have found that eNOS is closely related to hypertension, kidney injury, etc. eNOS can make an decrease in PKM2, which is an important mediator for catalyzing glycolysis and increasing the conversion of phosphoenolpyruvate (PEP) to pyruvate. The pyruvate produced by glycolysis is the raw material for aerobic and anaerobic respiration in cells. Once the ability to generate pyruvate weakens, the energy metabolism pathway of glucose will be greatly weakened. In addition, under disease conditions, the production of ischemia and hypoxia enhances the glycolytic pathway and leads to an imbalance of redox reactions. Further exacerbating inflammation and fibrosis. TGF β1, CTGF, TNF-α, and other factors are upregulated, further exacerbating renal injury. The role of inflammation and fibrosis in hypertensive renal injury is well known. Many studies have pointed out that hypertension is considered a systemic inflammatory disease, which is the aggregation of pro-inflammatory factors and inflammatory cells [20–22]. Their aggregation can lead to cell proliferation, glomerulosclerosis, and renal interstitial fibrosis. Experiments [22] have confirmed that the use of cell proliferation inhibiting drugs in patients with hypertensive renal damage can slow down or even reverse the process of kidney damage. Hypertensive renal injury is an inflammatory injury caused by the interaction of multiple immune cells, cytokines, and chemokines. Macrophages and T lymphocytes produce pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-17 (IL-17), and TNF-α to promote

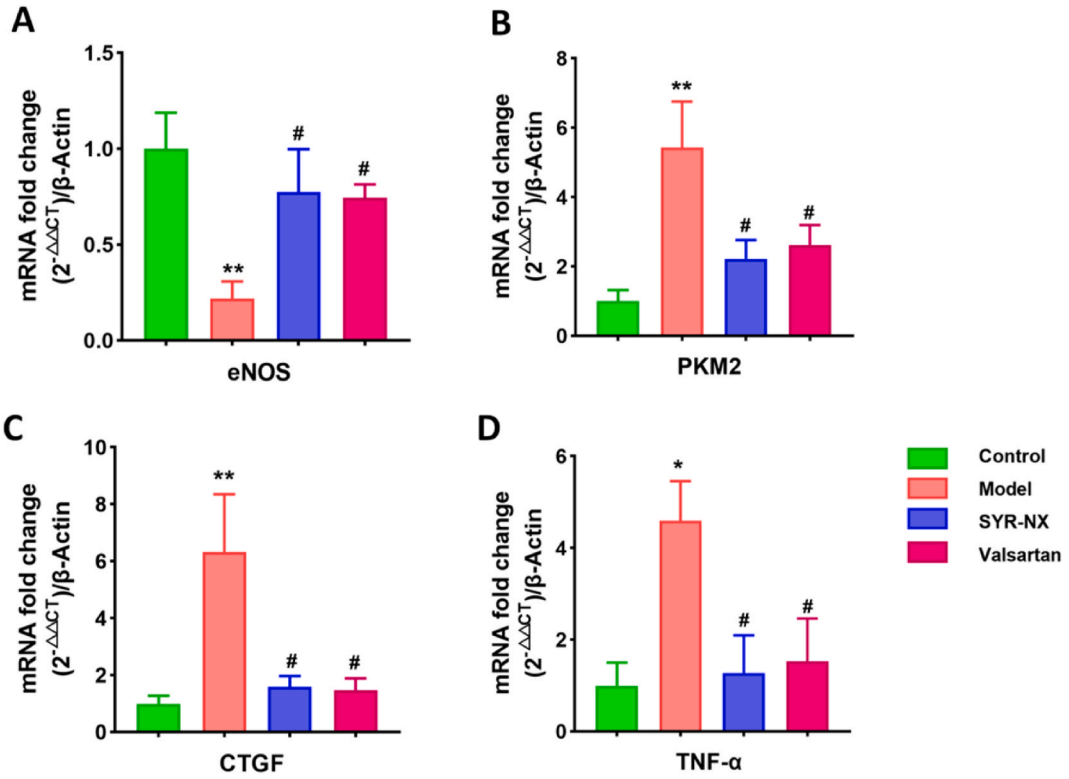

Fig. 6. Effect of SYR-NX on eNOS (A), PKM2 (B), CTGF (C), TNF-α (D) of Hypoxia induced HK2 cells examined by using qPCR. $^{*}P < 0.05$ $^{**}P < 0.01$ vs WKY, $^{\#}P < 0.05$ vs model group.

inflammation with chemokines and adhesion molecules together, while B lymphocytes and dendritic cells regulate blood pressure by promoting T cell activation, indirectly participating in the progression of hypertensive renal damage. Additionally, TGF-β1, CTGF and other classic inflammatory factors are activated and participate in the entire process of hypertension. At the same time, they interact with each other and jointly participate in renal cell proliferation. In summary, eNOS can alter metabolic pathways, leading to imbalanced oxidation and exacerbating inflammation and fibrosis, thereby affecting renal injury.

Our previous experiments have shown that the methods of clearing the liver, tonifying the kidney, and removing blood stasis and unblocking collaterals can alleviate kidney damage in hypertension through antioxidant, anti-inflammatory, and anti fibrotic effects [9–15]. SYR-NX have good clinical efficacy in treating hypertensive kidney damage by clearing the liver, tonifying the kidney, and removing blood stasis and unblocking collaterals. Cornus officinalis is sour, astringent, slightly moisturizing, nourishing the liver and kidneys, and can also astringent the essence. Its main active ingredients are iridoids, triterpenes, flavonoids, tannins, organic acids, polysaccharides, etc., which are closely related to energy metabolism and can play an antioxidant role in energy metabolism [35]. Radix achyranthes bidentata has a sweet and slightly bitter taste, which belongs to the liver and kidney meridians. Its can clear liver heat and calm liver yang. The main components of Radix achyranthes bidentata include saponins and sterone compounds, which can

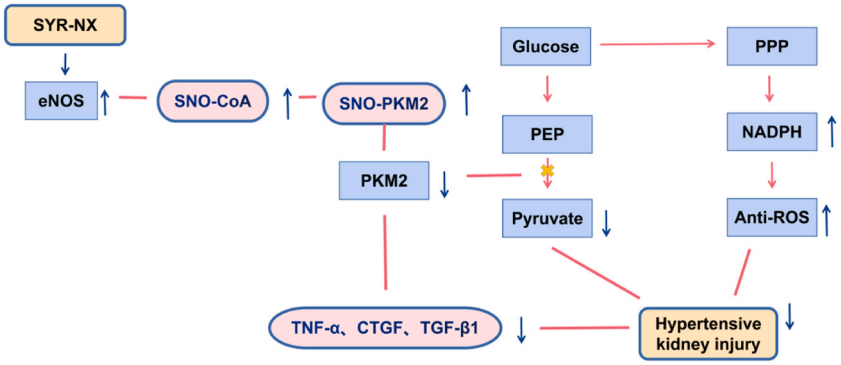

Fig. 7. Schematic diagram of metabolic reprogramming of hypertensive kidney injury promoted by eNOS with SYR-NX.

significantly improve energy metabolism, inflammation and fibrosis. In recent years, traditional Chinese medicine formulas mainly composed of SYR-NX have been widely used and have significant therapeutic effects in the treatment of various chronic kidney diseases, especially in the case of blood stasis and heat syndrome with obvious urinary protein. However, the pharmacological mechanism has not been fully elucidated. This study found that the main chemical components of SYR-NX are Asiatic acid, Gallic acid, and Oleanolic acid, which were predicted to be closely related to e-NOS using computers. We found that SYR-NX can inhibit renal cell oxidation, inflammation and fibrosis by regulating the eNOS related pathway, upregulate eNOS activity, inhibit PKM2, reduce glycolysis, lower pyruvate and lactate, increase ATP, and thereby inhibit renal cytokine TNF-α and CTGF to improve hypertensive kidney injury. The schematic diagram of metabolic reprogramming of hypertensive kidney injury promoted by eNOS with SYR-NX is shown in Fig. 7. Due to time constraints, gene knockdown was not performed, and metabolic indicators were still relatively limited. In the future, there is an opportunity to further explore more relevant indicators, and combine proteomics, metabolomics/immunofluorescence and other methods to further explore the effects of SYR-NX on hypertensive renal damage. For the intervention of early renal injury in patients with hypertension, different doses of SYR-NX may have different effects. In the future, it is necessary to further use different doses of SYR-NX to treat hypertensive renal injury and observe its therapeutic effects. Our current findings and discussions are preliminary, and are only a small part of the treatment of hypertensive renal injury with traditional Chinese medicine. We hope that Chinese medicine scholars can keep up with the times and continuously explore the internal mechanisms of disease prevention and treatment with traditional Chinese medicine, so as to make more contributions to human health.

5. Conclusions

ENOS is closely related to hypertension and renal injury, and has a protective effect on hypertensive renal injury. The present study demonstrated the effects of SYR-NX on hypertensive kidney injury and the underlying mechanism related to eNOS. SYR-NX increased the expression of eNOS, NADPH and ATP, reduced the expression of PKM2, pyruvate, lactate, CTGF and TNF-α by regulate metabolic reprogramming to improve inflammation and fibrosis to promote hypertensive kidney injury. In total, our study provides the basis for the treatment of hypertensive kidney injury with SYR-NX. Meanwhile, more explorations are needed to standardize the dose of SYR-NX. Our current study is preliminary. More depth researches should be performed to validate our findings.

Ethics approval

This experiment was approved by the Ethics Committee of Experimental Animal Research of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Animal Ethics No.: 2021 DW-07-02).

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Data availability statement

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

CRediT authorship contribution statement

Lichao Qian: Conceptualization. **Zhongchi Xu:** Writing – original draft, Formal analysis, Data curation. **Yanran Chen:** Visualization, Methodology, Data curation. **Zhao Gao:** Writing – original draft, Investigation, Data curation, Conceptualization. **Tianjiong Luo:** Methodology, Formal analysis, Data curation, Conceptualization. **Lihua Wu:** Supervision, Software, Resources, Project administration. **Yawei Zheng:** Writing – review & editing, Visualization, Supervision, Software. **Li Chen:** Resources, Project administration, Methodology, Investigation, Data curation. **Dongping Yuan:** Visualization, Validation. **Shuai Ren:** Writing – review & editing, Visualization, Validation, Supervision. **Yinxing Zhu:** Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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