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Renoprotective Effect and Mechanism of Qizhi Zhenwu Decoction in Murine Models of Doca-Salt Hypertension

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Abstract: The purpose of this study was to evaluate the renal protective effect of Qizhi Zhenwu Decoction on rats with DOCA salt hypertension and its influencing mechanism. In this study, the left kidney of rats was excised, and deoxycorticosterone acetate (DOCA) was injected under the epidermis, and a rat model of DOCA salt hypertension was made. They were then randomly divided into five groups: sham group, model group, Spironolactone group (Spi), QZZWD low-dose group (QZL), and QZZWD high-dose group (QZH), each group containing 7 rats. Several physiological measurements were done as follows: After two weeks of drug intervention, the blood pressure of mice was measured by tail sleeve method. Reactive oxygen species (ROS) were determined by fluorescence staining. Blood urea nitrogen and creatinine were detected by intelligent biochemical analyzer. Urine protein was detected by biochemical kit; The gene expression levels of NOX2, p47phox, NF-KB-p65, TGF-, TNF- and IL-6 were detected by western blotting. Compared with the sham operation group, the DOCA salt group showed obvious advantages compared with the model reference group in the gene expression levels of urinary protein, constriction pressure, urea nitrogen, creatinine, ROS and TGF-β, NOX2, P-NF-KB-P65, p47phox, TNF-αand IL-6 ($p<0.05$). QZZWD may maintain renal function by restricting NOX2/ROS/NF-KB signaling channels.

1. Introduction

By 2025, the global incidence of hypertension is expected to increase to 60 percent, which implies an estimated 1.56 billion patients. In the USA, 45% of people have hypertension, and the prevalence of high blood pressure surges with age. Nowadays, fighting back hypertension, as well as limiting hypertensive target organ damage, has already become a major clinical concern. Clinically, the kidney is a common target of hypertension that can easily incur organ damage. On the one hand, the kidneys regulate circulation by regulating the balance of water and electrolytes (especially sodium), which has a positive effect on blood pressure. Vascular constriction, on the other hand, can be regulated by the renin-angiotensin system. Failure to continuously regulate hypertension, resulting in renal dysfunction, may result in end-stage renal disease. Therefore, preservation of kidney function is key to preventing hypertensive target organ damage to the kidney.

In recent days, with the fast development of Chinese medicine in China, the clinical efficacy of

traditional Chinese medicine (TCM) in preventing hypertensive target organ damage has been widely noticed. Therefore, this study is aimed to figure out the renoprotective effect of QZZWD on hypertension and its specific mechanism using a DOCA-salt hypertensive murine model.

2. Materials and Methods

2.1 Animals and Medicines

In this study, 35 male Sprague-Dawley (SD) rats, with a growth cycle of six to seven weeks and an approximate weight of 19,010 g, were purchased from Chengdu Dashuo Experimental Animal Group (License number: SCXK (Sichuan) 2020-030). All experimental animals were adaptively housed under 12-h day/night alternation at 22-26℃ with a humidity of 40%-50% for 7 days before experimentation.

QZZWD is made of blast-fried aconite (9g), Atractylodes macrocephala (12g), White peony (18g), Ginger (18g), Poria cocos (18g), leeches (6g), Astragalus (30g). The raw materials are mainly selected from Shaanxi University of Chinese Medicine Hospital. It is cooked in ultra-pure water.The aconite was decocted for 40 mins first, and the rest of the drugs were soaked in cold water for 20 mins. They were then put into the aconite decoction and decoted for 1.5 h to get the first filtrate; the remaining residue is then cooked for an hour and a half to obtain a second filtrate; Mix the two filtrates to get the final decoction.The liquid of QZZWD was concentrated to 1.11g/ml (QZL group) and 2.22g/ml (QZH group), respectively. Spironolactone tablets are mainly purchased from Hangzhou Minsheng Pharmaceutical Company (Batch number: H33020070; specification: 20mg/tablet). Deoxycorticosterone acetate (DOCA) was mainly purchased from Shanghai McLean Biochemical Technology Group (Approval Permit No: D830008; specification: 1g).

2.2 Modeling Method

SD rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium. The dorsal skin area of the rats was completely shaved in a prone position.After disinfection, an approximately 2cm incision was made at the lower costal margin of the left spina bifida for the later separation of the muscles. The renal capsule was then removed before the left kidney was able to be exposed. After ligation of renal veins and arteries near the hilum, the left kidney was excised. The final operation is performed to remove and close the congestion; At the same time, penicillin sodium was injected intraperitoneally for 3 days after operation. Sham animals were treated by the same method, but no ligation or excision of the left kidney was performed. DOCA-salt rats received subcutaneous injections of DOCA (120 mg/kg, dissolved in dimethylformamide) twice a week for four weeks with high salt water (1% NaCl) [1]. The sham group received subcutaneous injections of an equivalent volume of dimethylformamide solution, at an identical frequency for four weeks, and with distilled water.

2.3 Grouping and Intervention Methods

The rats were randomly divided into five groups: sham operation group, model group, positive comparison group, QZZWD low dosage group (QZL), QZZWD high dosage group (QZH), (spironolactone group or Spi), and seven rats were allocated to each group. The intervention patterns of each group are shown below.

Sham group: subcutaneous injections of dimethylformamide twice a week for a month; the rats were given distilled water intragastrically for four weeks; distilled water was chosen for daily drinking.

Model group: subcutaneous injections of DOCA twice a week for 4 weeks; The rats were given distilled water intragastrically for two weeks; 1% high salt water was chosen for daily drinking.

Spi: subcutaneous injections of DOCA twice a week for 4 weeks; rats were given 5mg/kg spironolactone daily for two weeks; 1% high salt water was chosen for daily drinking.

QZL: subcutaneous injections of DOCA twice a week for 4 weeks; the rats were given 11.1g/kg QZZWD daily for two weeks; 1% high salt water was chosen for daily drinking.

QZH: subcutaneous injections of DOCA twice a week for 4 weeks; the rats were given 22.2g/kg QZZWD daily for two weeks; 1% high salt water was chosen for daily drinking.

2.4 Sample Collection and Preservation

After 12 hours of anhydrous feeding, the rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30mg/kg). For each rat, blood was collected from the abdominal aorta. Blood samples were collected and centrifuged at 3000R/min for 10min at normal temperature. Serum and plasma were separated from each other, and then the separated samples were stored in a refrigerator at -80℃ for 30min. After excision of the right kidney, the rats were inactivated through cervical dislocation. The kidneys were weighed, then washed with phosphate buffered saline (PBS), and finally drained with filter paper. The renal capsule was avulsed, and then divided into two halves along the paramedian coronal surface. After they were rinsed clean with PBS, one half was put in 4% paraformaldehyde fixative solution, dehydrated after 24h, embedded in paraffin, and put in a 4℃ freezer, while the other half was put into an EP tube, and frozen at a -80℃ freezer.

2.5 Measurement Method

2.5.1 Arterial Bp Measurement by the Tail-Cuff Method

After one week of adaptive feeding, the arterial BP was monitored by the tail artery manometry method in the first, fourth, and eighth weeks.

2.5.2 Fluorescence Staining

The renal tissues of rats were treated into 10 um thick frozen sections, on which the clearing solution was spread and then removed. The GENMED staining solution (DCFH-DA) was added dropwise to the sections, before they were incubated at 37℃ for 20 minutes. Then eliminate DCFH-DA, at the same time the elimination solvent on the corresponding. Afterwards, the sections were covered with slides and put under a fluorescence miscroscope for the observation of their fluorescence intensity. They were then photographed and scanned. Image Pro Plus is used as the integral optical density (IOD) of dissected sections, which can effectively illustrate the relative ROS content of kidney tissue.

2.5.3 Biochemical Testing

The serum separated from blood was obtained by centrifugation for the detection of creatinine and urea nitrogen using a fully automated biochemical analyzer. Before taking the materials, the rats were housed in metabolic cages for 24 hours, and were fed with water only. 24-hour urine sample was then collected and determined by Coomassie bright blue staining.

2.5.4 Western Blot

The gene expressions of NOX2, p-NF-KB-p65, p47phox, IL-6 and TGF-β were detected by

western blotting. To obtain the 0.2g renal tissue sample, RIPA lysis solution was added for the following 30-min lysis on ice. After centrifugation at 10000rpm at 4℃ for 10min, the supernatant was obtained and collected. Protein levels were determined using a BCA kit containing samples. The samples were electrophoretically processed by SDS-PAGE (80v to 100V) and transferred to PVDF membranes at a fixed current of 150mA. The PVDF membrane was then sealed in TBST with 5% dry milk for two hours, After washing the membrane with TBST, NOX_2, p47phox, NF-KB-p65, TGF-β, TNFα and IL-6 were added dropwise (diluted at 1:1000) to a membrane, Meanwhile, the membrane was incubated overnight at 4℃. The next day, TBST was used to flush the cells three times, and after the addition of HRP labeled second antibody, the cells were placed in a shaker at room temperature for one and a half hours. Then use ECL to stain the samples, and analyze the gray scale values of the protein bands by Image (software).

2.6 Statistical Method

SPSS 25.0 was used to analyze the data, and the statistical data were expressed by means of average value ± standard deviation. When the data presented a normal distribution and the variance was assumed to be uniform, the comparisons between multiple groups were analyzed using single index variance analysis and then detected using Fisher LSD. When the data did not follow the normal distribution, nonparametric detection was used to compare the data between multiple groups. Chi-square detection was used for data measurement. $P < 0.05$ meant statistical significance, $P < 0.01$ meant strong significance.

3. Results

3.1 Changes of Sbp in Rat Caudal Artery

As Table 1 below shows, there was no statistically significant difference in blood pressure before surgery among the five groups. After 4 weeks of gavage, compared with the sham group, the model group showed an obvious increase in the systolic BP of the rats (101.14±10.95 vs. 181.43±4.86g, $p<0.05$). Compared with that of the model group, the systolic BP of the rats in Spi, QZL and QZH decreased significantly (145.14±4.34, 123.86±6.64, 139.43±8.32 mmHg, $p < 0.05$). Compared with Spi, QZL showed more obvious effect on reducing blood pressure ($p < 0.05$) [2].

Table 1 Effect of Qzzwd on Systolic Bp of Doca -Salt Rats (Mmhg)

Group	Preoperative baseline BP	BP before treatment	BP after treatment
sham	98.14±14.57	99.14±14.93	101.14±10.95
model	101.29±7.18	166.29±9.71	181.43±4.86$^{\#}$
spironolactone	97.86±8.09	164.71±10,21	145.14±4.34$^{\#\&}$
Qizhi low-dose	97.57±13.96	165.14±8.71	123.86±6.64$^{\#\&*}$
Qizhi high-dose	97.71±8.92	165.14±9.35	139.43±8.32$^{\#\&}$

Note: #: compared with the sham group, $p<0.05$; &: compared with the model group, $p<0.05$; *: compared with Spi, $p<0.05$.

3.2 The Results of Ros

ROS were important participants in oxidative stress responses and they promoted vascular endothelial cells to produce intercellular adhesion molecules, leading to inflammatory cell infiltration and aggravated inflammatory responses [3]. As shown in Figure 1 below, ROS content in kidney of the model group was significantly increased compared with the sham operation group (p

< 0.05). Compared with model group, Spi, QZL and QZH could reduce ROS gene expression in serum, and the difference was significant ($p < 0.01$).

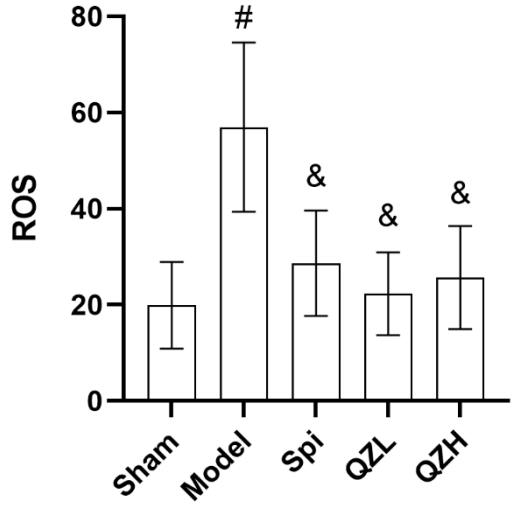

Fig.1 Comparison of the Ros Levels in Each Group

Note: #: Compared with the Sham Group, P<0.05; &: Compared with the Model Group, P<0.05; *: Compared with Spi, P<0.05.

3.3 Comparison of the Levels of Creatinine, Urea Nitrogen, and Urinary Protein

The levels of creatinine (SCR) and urea nitrogen (BUN) can reflect the filtration function of the kidney and the severity of renal contusion [4]. An experimental study showed that QZZWD significantly reduced creatinine and urea nitrogen content in mice with DOCA saline hypertension, which proved that QZZWD can effectively improve the filtration performance of kidney tissues and organs.The content of urinary protein can reflect the reabsorptive capacity of renal tubules and glomerular filtration, which was an important sign of renal injury [5]. As shown in Table 2, compared with the sham group, the model group showed obvious higher levels of BUN, CRE and urinary protein in the serum ($p<0.05$). Compared with model group, BUN, CRE and urinary protein contents of Spi, QZH and QZL were significantly decreased ($p < 0.05$). Compared with Spi, the urinary protein content in QZL was significantly decreased ($p > 0.05$). The results showed that QZZWD had obvious protective effect on renal contusion in mice with DOCA salt hypertension.

Table 2 Effects of Qzzwd on the Content of Creatinine and Urea Nitrogen and Urinary Protein in Doca-Salt Rats

group	creatinine (μmol/L)	urea nitrogen (mmol/L)	urinary protein (mg)
sham	25.85±5.60	33.10±2.48	27.76±9.07
model	56.34±6.00$^{\#}$	43.50±2.87$^{\#}$	142.05±27.72$^{\#}$
spironolactone	37.72±3.03$^{\#\&}$	37.98±3.18$^{\#\&}$	82.47±7.22$^{\#\&}$
Qizhi low-dose	34.90±4.55$^{\#\&}$	39.16±2.76$^{\#\&}$	42.09±11.60$^{\&*}$
Qizhi high-dose	42.58±4.00$^{\#\&}$	38.62±2.83$^{\#\&}$	64.95±11.27$^{\#\&}$

Note: #: compared with the sham group, p<0.05; &: compared with the model group, p<0.05; *: compared with Spi, p<0.05.

3.4 Results of Western Blot

As shown in Figure 2 below, compared with the sham operation group, gene expressions of NOX2, p-NF-KB-P65, p47phox, TGF-β, TNF-α and IL-6 in the model group were significantly increased ($p<0.05$). Compared with the model group, QZH, Spi and QZL could reduce the expression level of P-NF-KB-P65, p47phox, transforming growth fact-β, Spi and QZL NOX2 ($p<0.05$). Compared with that of Spi, the effect of the inhibition of the synthesis of IL-6, p-NF-kB-p65, TGF-β, and TNF-α in QZL was significant ($p<0.05$). Lambeth found that the occurrence of chronic diseases such as hypertension was related to the excessive production of ROS by NOX enzymes [6]. Previous studies have found that ROS can induce the phosphorylation of NF-kB p65, activate NF-kB, and then induce inflammatory injury [7].

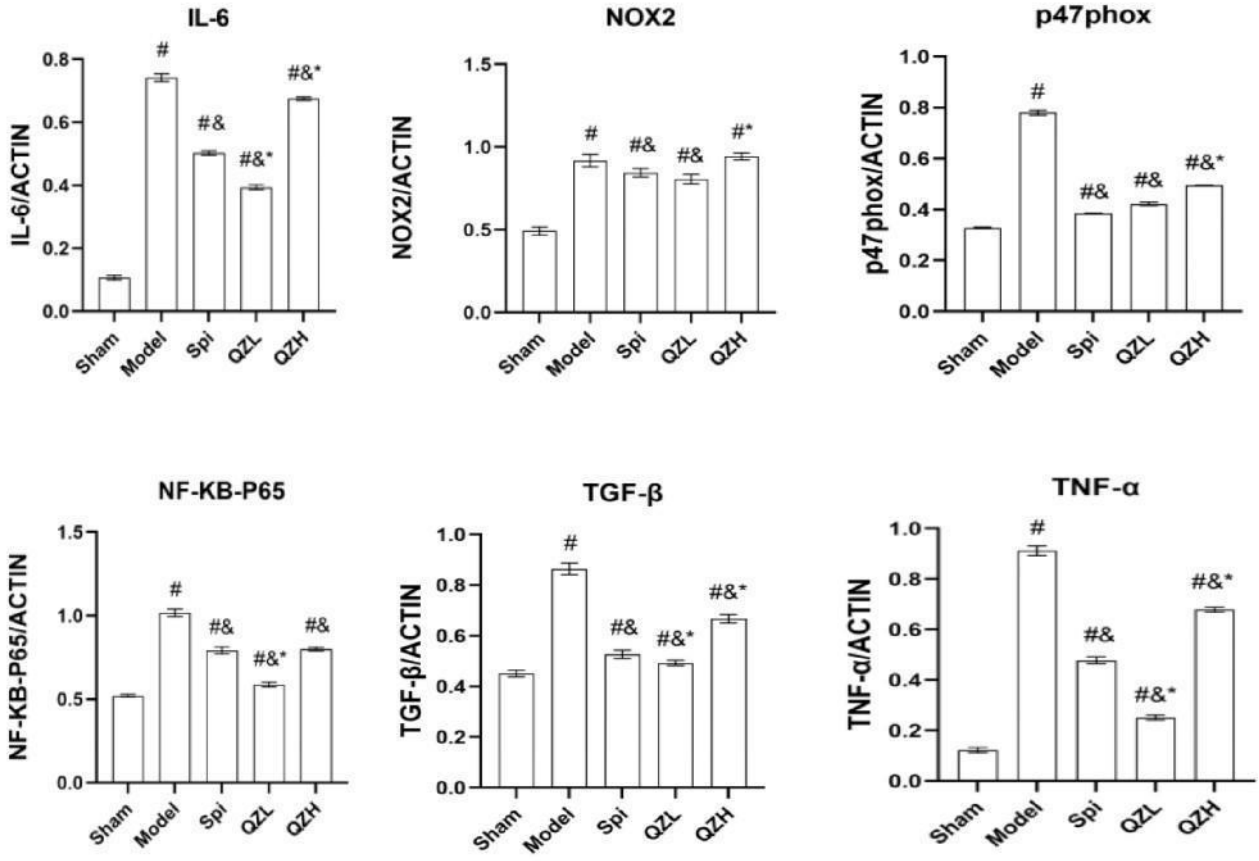

Figure 2. Comparison of the results of blotting data in different groups of rats

Note: #: compared with the sham group, $p<0.05$; &: compared with the model group, $p<0.05$; *: compared with Spi, $p<0.05$.

4. Discussion

DOCA salt hypertension rat model (DHR) was constructed by subcutaneous injection of DOCA and high saline water. The results showed that the BP increased and the proteinuria result was positive, which verified the success of membrane formation.

The results of this study confirmed that compared with the model group, the blood pressure of rats in the QZL, QZH and Spi groups was significantly reduced. Therefore, QZZWD significantly improved the BP of rats with hypertensive renal injury, so as to preserve their renal functions. The levels of urea nitrogen (BUN) and creatinine (SCR) can effectively reflect the filtration function of

the kidney and the severity of kidney damage. An experimental study showed that QZZWD can significantly reduce the gene expression of creatinine and urea nitrogen in rats with DOCA salt hypertension, indicating that QZZWD can effectively improve the filtration function of kidney tissue. The content of urinary protein can reflect the reabsorptive capacity of renal tubules and glomerular filtration, which was an important sign of renal injury. QZZWD could reduce 24-h urinary protein in rats with hypertensive renal injury, which indicated that it could improve renal tubular reabsorption and glomerular filtration function.

ROS were important participants in oxidative stress responses and they promoted vascular endothelial cells to produce intercellular adhesion molecules, leading to inflammatory cell infiltration and aggravated inflammatory responses. The results showed that QZZWD could down regulate the level of ROS in renal tissues of rats with hypertensive renal injury and protect the kidney. NADPH oxidase family is essential origin of ROS generation, and NOX_2 is an important member of NOX family. P47phox is a class of NOX2 cytoplasmic subunits. According to the results of this study, the gene expression degree of NOX2 and p47phox in the QZZWD diagnosis and treatment group was significantly lower than that in the model group ($p<0.05$), while the ROS content was lower than that in the model group. Therefore, QZZWD regulated oxidative stress in rats with renal injury by inhibiting the NOX2-ROS pathway. NF-kB is a class of nuclear transcription genes that initiate and regulate inflammatory disease responses and mediate tissue contusion. P65 is a common subunit of NF-kB that exists specifically as a heterodimer. The results of this experiment showed that the gene expressions of NF-kB, TNF-, TGF- and IL-6 in the QZZWD treatment group were significantly lower than those in the model group ($p<0.05$).Therefore, QZZWD not only reduced the ROS level of DHR, but also inhibited the activating of NF-kB, reduced the protein expression of inflammatory factors. Therefore, QZZWD can reduce oxidative stress and inflammatory disease bruising by restricting NOX2/ROS/NF-KB signaling channel, and effectively protect kidney tissues and organs.

To sum up, Qizhi Zhenwu Decoction has the effect of lowering blood pressure and adjusting kidney tissue function. The mechanism may be related to the adjustment of NOX2/ROS/NF-KB channels.

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