Protective effects of astragaloside in rats with adriamycin nephropathy and underlying mechanism

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Available online 20 Apr., 2016

[ABSTRACT] The present study was designed to determine the mechanism underlying the treatment of nephrotic syndrome using astragaloside by observing the effects of astragaloside on the expression of nephrin and podocin proteins and genes in kidneys of rats with adriamycin nephropathy. The rats were injected with adriamycin and, after successful model establishment, randomly divided into a model group, a Methylprednisolone (MP) group, and an astragaloside group. The 24-h complete urine samples were collected. Biochemical indicators were monitored, and kidney tissues were collected for pathological analysis using light microscopy and electron microscopy. The mRNA expression of nephrin and podocin was measured in the kidney tissues using the real-time qPCR, and the protein expression levels of nephrin and podocin were detected using Western blot analysis. At the end of 12 weeks of drug intervention, the urinary protein level was lower in the MP and astragaloside groups than that in the model group (P = 0.008 and P = 0.01, respectively). Serum albumin was higher in the MP and astragaloside groups than in the model group (P < 0.001 and P = 0.012, respectively). Podocytes in the MP group were nearly normal, and fusion of podocytes in the astragaloside group was significantly less than that in the control group. The nephrin and podocin mRNA and protein expression levels in the intervention groups were higher (P < 0.05) than that in the model group. Due to the increased expression of podocyte-related nephrin and podocin proteins, astragaloside maintained slit diaphragm integrity and decreased the level of proteinuria in rats with adriamycin nephropathy.

[KEY WORDS] Astragaloside; Adriamycin nephropathy; Podocyte; Nephrin; Podocin

[CLC Number] R965

[Introduction] Nephrotic syndrome (NS) is a commonly seen kidney disease in clinical practice. Minimal change nephrotic syndrome (MCNS) is one of several pathological types and exhibits clinical manifestations, such as heavy proteinuria, hypoproteinemia, hyperlipidemia, and edema [1-2]. Studies have shown that the development of proteinuria is closely associated with the glomerular filtration membrane, which comprises vascular endothelial cells, glomerular basement membrane (GBM), and podocytes (foot processes) [3]. The slit diaphragm (SD), which occurs between the foot processes of podocytes, is the most important component to maintain the integrity of the glomerular filtration membrane [4]. It has been shown that the SD is composed of many protein molecules, including nephrin, podocin, CD2 adapter protein (CD2AP), zonula occludens (ZO-1), FAT and transient receptor potential canonical 6 (TRPC6) [3]. When it is destroyed, small protein molecules will be filtered out at this point, causing proteinuria [4]. The Adriamycin (ADR)-induced NS, which is first reported by Bertani et al., is a classical NS model. Adriamycin induces thinning of the glomerular endothelium and podocyte effacement associated with loss of size- and charge-specific barrier to filtration of plasma proteins [3]. Renal function is also affected, with rodents showing reduced serum albumin, dyslipidaemia and increased urine protein excretion consistent with the NS [6].

In recent years, immunosuppressants (steroid, cyclosporine A, etc.) have been used based on anecdotal evidence to treat...
NS [7]. Adults with NS caused by MCNS continue to pose a challenge to clinicians. Unless a contraindication exists, corticosteroids continue to be first-line therapy [8]. Methylprednisolone (MP) is one of the corticosteroids, which is widely used to treat NS [8]. Shinzawa et al. have shown that use of MP is associated with earlier remission and lower incidence of relapse in NS caused by MCNS [9]. However, immunosuppressant therapy can induce many serious side effects, such as diverse organ failure (cardiac, renal, and ear), toxicities, fungal infection, and a fast relapse following any effects, such as diverse organ failure (cardiac, renal, and ear). Therefore, research on safer and more effective drugs is necessary. We use the adriamycin nephropathy animal model, which was prepared using a single injection of adriamycin into the tail vein. Intervention treatment using astragalosides was conducted to observe the effect of astragalosides on the glomerular filtration barrier in adriamycin nephropathy animal model, which was prepared using a single injection of adriamycin into the tail vein. Intervention treatment using astragalosides was conducted to observe the effect of astragalosides on the glomerular filtration barrier in adriamycin nephropathy animal model and the underlying mechanism of action.

Materials and Methods

Experimental animals
Fifty healthy, clean grade, male Wistar rats weighing 180–200 g, were purchased from Vital River Laboratories (Beijing, China) (license number SCK (Jing) 2012-0001, approval date: March 1st 2015). The rats were acclimated for 1 week in the clean-grade animal room of the Experimental Animal Center in the PLA General Hospital, Beijing, China. The rats were housed in an air-conditioned room at 25 ± 2 °C and 65% humidity, with a 12 h light/12 h dark cycle. All animal experiment procedures were carried out in accordance to protocols approved by the Ethics Committee for Animal Experimentation of PLA General Hospital and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs
MP was purchased from Pfizer Inc. (Lot No. V854A, New York, USA); Adriamycin hydrochloride was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. (Lot No. 44583, Shanghai, China), and astragaloside was purchased from Shaanxi Scipharm Biotechnology Co., Ltd. (Lot No. 201402025, Xi’an China).

Major instruments and reagents
Nephrin and podocin primary antibodies were purchased from Abcam Co., Ltd. (Lot No. GR 101789-4 and GR 166437-2, London, UK), goat anti-rabbit antibody was purchased from Beyotime Co., Ltd. (Lot No. A0208, Shanghai, China), and anti-β-actin antibody was purchased from Santa Cruz (California, USA). Trizol reagent was purchased from Ambion Co., Ltd. (Lot No. 87701, Shanghai, China). Nephrin Taqman and podocin Taqman were purchased from ABI (Lot No. P141201 and 1341570, Texas, USA), and EasyScript First-Strand cDNA Synthesis was purchased from TransGen Biotech (Lot No. #I20619, Beijing, China).

Preparation of the animal model
The rats were given routine feed and water ad libitum for adaptive feeding for continuous 7 days before the model was induced. Ten of the 50 rats were randomly selected as the control group rats. The remaining 40 rats were anesthetized using sodium pentobarbital (30 mg·kg⁻¹) and immobilized on a rat platform at a supine position. The tails of the rats were disinfected using 70% ethanol, and adriamycin hydrochloride (5 mg·kg⁻¹) was injected once into a tail vein to prepare the adriamycin nephropathy model [5]. The control group rats were injected with normal saline via a tail vein (5 mL·kg⁻¹). After 2 weeks of model establishment, 24-h urinary protein was quantified. If the amount of 24-h urinary protein was higher than 50 mg, the model was considered to have been established successfully. Using this procedure, the model was successfully established in 33 rats. Thirty rats were randomly divided into a model group, a MP group, and an astragaloside group, with each group comprising 10 animals.

Drug intervention
After the model had been established successfully, intragastric administration was performed for 3 months in all the experimental and control groups. In the normal control and model control groups, normal saline was administered at 5 mL·kg⁻¹·d⁻¹. In the MP group, MP dissolved in normal saline was administered at 20 mg·kg⁻¹·d⁻¹, and in the astragaloside group, astragaloside dissolved in normal saline was administered at 150 mg·kg⁻¹·d⁻¹.

Urinary examination
The 24-h complete urine samples were collected at various time points i.e., before model establishment, 2 weeks after model establishment, and 4, 8, and 12 weeks after intervention. Each rat was placed in a metabolic cage for 24 h. The animals were fasted starting from 12 h before urine collection until the end of urine collection (the animals were allowed free access to water). After the urine samples were collected, the 24-h urine volume was recorded. The concentration of urinary protein was measured using a
Mindary BS480 analyzer, and the total 24-h amount of urinary protein was calculated.

**Blood biochemical tests**

At the end of 12 weeks of drug intervention, the rats were sacrificed by dislocation of the spine; blood samples were collected from the inferior vena cava. Serum creatinine (Scr), blood urea nitrogen (BUN), albumin (ALB), total cholesterol (CHO), triglycerides (TRIG), and uric acid (UA) were measured using a COBAS701 biochemical analyzer.

**Examination of kidney pathology using light microscopy**

After conventionally paraffin-embedding the rat kidneys, tissue blocks were cut parallel to the short axis of kidneys through the renal hilum into 4-µm sections. The sections containing whole cross sections of kidney were chosen for periodic acid-Schiff staining, and pathomorphological changes were observed under a light microscope.

**Examination of kidney ultrastructure using electron microscopy**

Renal cortex was fixed in phosphate buffer (pH 7.2) containing 3% glutaraldehyde and 0.22 mmol·L⁻¹ sucrose. After fixing in 1% osmium tetroxide, the samples were dehydrated in an ethanol gradient and embedded in epoxy resin. The pathology of the kidney ultrastructure was examined using a Hitachi H-600 transmission electron microscope.

**Western blot analysis**

The expression levels of nephrin and podocin proteins were detected using Western blots. Total protein was extracted from renal cortical tissues using RIPA protein extraction buffer containing protease inhibitors and PMSF. Protein concentrations were determined using the bicinchoninic acid (BCA) method. SDS-PAGE was performed using 100 µg total protein in each lane at a constant 100 mA current. Protein samples were wet-transferred onto a PVDF membrane, which was incubated with primary antibodies against nephrin (1 : 2 500), podocin (1 : 2 000), or β-actin (1 : 1 000) at 4 ºC overnight. After washing with TBST, the membrane was incubated with the secondary antibody at 37 ºC for 60 min. After washing with TBST, the protein bands were gradient-exposed in a dark room. The results were analyzed using ImageJ software (National Institutes of Health), and β-actin was used as a loading control.

**Real-time fluorescence quantitative PCR detection**

The total RNAs from different experimental groups were obtained by Trizol method. The concentration of RNA was determined by an absorbance at 260 nm and the purity of the RNA was evaluated by measuring the A260/A280 ratio. Reverse transcription was performed using 10 µL of 2*ES Reaction Mix, 1 µL of Enzyme Mix, 1 µL of Oligo dT Primer, and RNA template; the total volume was increased to 20 µL using RNase-free water. The reaction conditions were 37 ºC for 30 min and 85 ºC for 5 min. Real-time PCR was performed using the Taqman probe method. The real-time fluorescence quantitative PCR reaction was performed in a 20 µL volume including 4 µL of cDNA sample, 10 µL of Mix solution, 5 µL of ddH2O, and 1 µL of Taqman. The real-time quantitative PCR used ABI7900. All real-time fluorescence quantitative PCR experiments were performed strictly according to the MIQE(Minimum Information for Publication of Quantitative Real-Time PCR Experiments).

**Statistical analysis**

Statistical analyses were performed using SPSS 17.0 software (Statistical Product and Service Solutions). The measurement data are presented as means ± SD. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). Comparisons between two groups were performed using the LST test. *P* < 0.05 indicated statistically significant.

**Results**

**Changes in body weight**

Compared to the control group, the mean body weight increased slowly in the model group. By the end of the 12-week intervention, the body weight of the rats in the model group significantly decreased (*P* < 0.01). The mean body weights were higher in the MP and astragaloside groups than that in the model group (*P* < 0.01, *P* = 0.002, respectively). The mean body weights were not statistically different between the MP group and the astragaloside group (*P* = 0.057; Fig. 1).

![Fig. 1](image)

**Fig. 1** Comparisons of body weights among all Groups. Asterisks and the pound sign designate significant differences. *P* < 0.05 vs the model group. **P* < 0.01 vs the control group. #*P* < 0.01 vs the model group

**Quantitation of 24-h urinary protein**

Urinary proteins levels in the rats in each group were all at the same baseline level before model establishment. Two weeks after model establishment, the urinary protein of the rats increased. After 4 weeks of intervention, the urinary protein level in the MP group was lower than that in the model group (*P* = 0.028). The urinary protein level in the astragaloside group exhibited a decreasing trend, but this did not reach statistical significance. After 8 weeks of intervention, the urinary protein levels in the MP and
astragaloside groups were both lower than that in the model group (P = 0.009 and P = 0.033, respectively). After 12 weeks of intervention, the urinary protein levels were significantly higher in the model group than that in the normal control group (P = 0.002). Urinary protein levels were lower in the MP and astragaloside groups than that in the model group (P = 0.008 and P = 0.01, respectively). The differences were identified between the MP and astragaloside groups (P = 0.038; Fig. 2).

**Serum ALB, CHO, TG, BUN, CR, and UA after 12 weeks of intervention**

Compared to levels in the blank control, serum CHO (P = 0.001) and TG (P = 0.021) were higher and ALB was significantly lower (P < 0.001) in the model group. These results were consistent with the clinical symptoms of NS. Compared to the levels in the model group, serum ALB was higher in the MP and astragaloside groups (P < 0.001 and P = 0.012, respectively) and serum CHO (P = 0.035) and TG (P = 0.002) were lower in the astragaloside group. Compared to the levels in the MP group, TG was lower in the astragaloside group (P = 0.004). The levels of Scr, BUN, and UA did not significantly differ among all the groups (Table 1).

**Pathomorphological changes in kidney tissues**

Kidney tissues in the control rats presented no obvious lesions. Some rats in the model group had a mild focal segmental increase in mesangial cells and matrix, but no necrosis, crescent, and sclerosis were observed. Some focal granular and vacuolar degeneration was observed in tubular epithelial cells. There was no obvious tubular atrophy, and protein casts were occasionally observed in tubules. Focal inflammatory cell infiltration in the renal interstitium combined with a small amount of fibrous tissue proliferation was observed. Intimal thickening of some arterioles was observed. Pathological symptoms were less and inflammatory cell infiltration in the renal interstitium was less in the MP and astragaloside groups than that in the model group (Fig. 3).

![Fig. 3](image-url) Renal tissues viewed under a light microscope (periodic acid–Schiff (PAS) stain, × 400). A: control group; B: model group; C: MP group; D: astragaloside group

**Pathological changes in kidney ultrastructure by transmission electron microscopy**

The GBM in the normal control group was smooth, even, and without thickening; foot processes were clear and complete and were arranged in an orderly manner; no fusion occurred. The GBM in the model group was occasionally thickened. However, foot processes exhibited diffuse fusion and disappearance, representing a significant difference from that in the control group. These results confirmed that the model was successfully established. In the MP group, foot processes were clear and were similar to those in the control group. GBM in the astragaloside group was smooth and even;
foot process fusion was significantly lower, and only some fusion occurred; a few new vili were seen (Fig. 4).

**Changes in the expression of nephrin and podocin proteins in kidney tissues**

After 12 weeks of intervention, the expression of nephrin protein in the kidney tissues of rats in the model group was significantly lower than that in the normal control group ($P < 0.001$). Upregulation of nephrin protein expression in the MP and astragaloside groups was statistically different ($P = 0.004$ and $P = 0.042$, respectively) than that in the model group. Podocin protein expression in the kidney tissues of rats in the model group was significantly lower than that in the normal control group ($P < 0.001$). The expression of podocin protein in the MP and astragaloside groups was significantly higher than that in the model group ($P = 0.014$ and $P = 0.037$, respectively; Fig. 5).

![Fig. 4 Morphological changes in kidney podocytes under electron microscopy (×4200). A: control group; B: model group; C: MP group; D: astragaloside group](image)

![Fig. 5 Western blot analysis for nephrin, podocin and β-actin protein in renal tissues (A). Bar graphs representing the nephrin and podocin expressions relative densities to β-actin (B). Asterisks, the pound sign and triangle symbols designate significant differences. **$P < 0.01$ vs the control group. *$P < 0.05$, ##$P < 0.01$ vs the model group. △$P < 0.05$ vs the MP group](image)

**Expression of nephrin and podocin mRNAs in kidney tissues**

Genes that encode nephrin and podocin are important functional genes in podocytes and have been confirmed to play important roles in the production of urinary protein [4]. After 12 weeks of intervention, the expression levels of nephrin and podocin mRNA in the kidney tissues of rats in the model group were significantly lower than that in the control group ($P < 0.001$ and $P < 0.001$, respectively). The expression of nephrin mRNA in the MP and astragaloside groups was upregulated to different degrees, compared to the model group ($P < 0.001$ and $P = 0.007$, respectively). The expression of podocin mRNA in the MP and astragaloside groups was upregulated to different levels ($P < 0.001$ and $P < 0.001$, respectively); however, there was no significant difference between these two groups (Fig. 6).

**Discussion**

Currently, it is considered that proteinuria production is closely associated with structural or functional damage of the glomerular filtration barrier. NS, which represents glomerular filtration barrier damage, is an important clinical disease [2]. SD is an important component of the filtration...
Fig. 6 Bar graphs representing the nephrin, and podocin mRNA levels in the kidney tissue. Asterisks and the pound sign designate significant differences. **P < 0.01 vs the control group. ##P < 0.01 vs the model group

barrier and contains a variety of protein molecules [3]. Functional abnormality of SD proteins, such as podocin, nephrin, and TRPC6, will result in renal glomerular disease [14-16]. Studies have shown that in adriamycin nephropathy rats, nephrin plays important roles in the production of proteinuria and in the extension of GBM. Nephrin is the most sensitive molecule in this disease [17]. Injection of a monoclonal antibody (mAb 5-1-6 antigen) against SD in rats can cause proteinuria, indicating that in addition to congenital kidney diseases, nephrin also plays important roles in acquired kidney diseases [18]. Podocin is also a transmembrane protein and belongs to the stomatin family [19]. Podocin interacts with intracellular fragments of nephrin and CD2AP via its carboxyl terminus to promote or amplify nephrin-induced signal transduction [20]. The resulting change in podocin function will cause abnormal SD function [19]. Studies in animal models show that podocin-deficient rats produce a large amount of proteinuria and die of renal failure due to glomerular sclerosis [21]. Electron microscopy shows extensive podocyte fusion. The junctions of the remaining foot processes exhibited SD defects, the nephrin gene was downregulated [21].

The present study established an adriamycin nephropathy model by a single injection of 5 mg·kg⁻¹ adriamycin. The amount of urinary protein in the rat model group significantly increased. The amount of urinary protein continued to increase after 4, 8, and 12 weeks of model establishment. Plasma ALB significantly decreased. In addition, observation of glomerular podocytes in the rat model using electron microscopy showed obvious fusion of foot processes. The clinical pathological changes observed were similar to that observed in human NS [22], therefore, the model established was ideal for the experimental study. The protein and mRNA expression levels of nephrin and podocin in the model group significantly decreased, suggesting that podocin and nephrin played a definite role in the development of NS and in the production of proteinuria. The results suggest that the imbalance of nephrin and podocin expressions will lead to adysregulated ultrafiltration of glomerulus. Aastragaloside could restore the imbalances of the genes expression and prevent the podocytes from being injured. Body weight was lower in the model group than in the other groups; this observation might be associated with the significant decrease in plasma ALB and malnutrition.

In the present study, classical hormonal therapy [9] was used as a positive control to observe the therapeutic effects of astragaloside on adriamycin nephropathy and its molecular mechanism. Our results showed that intervention by astragaloside for 8 and 12 weeks reduced 24-h urinary protein. After 12 weeks, the serum ALB level in the astragaloside group was significantly higher than that in the model group. Observation of the glomerular podocytes by electron microscopy showed that the foot process fusion was less than that observed in the model group, indicating that astragaloside exerts a protective function on podocytes in rats with adriamycin nephropathy. The protein and mRNA expression levels of nephrin and podocin in the astragaloside group were higher by differing degrees than that in the model group. These results suggested that astragaloside might maintain the glomerular infiltration barrier by stabilizing podocin and nephrin proteins in the SD, thereby reducing urinary protein leakage. However, the effects of astragaloside on the duration and degree of reduction of proteinuria and on the relief of podocyte lesion in the kidney ultrastructure were weaker than those of MP. In addition, the upregulation of nephrin protein and mRNA expressions differed significantly. These results suggested that the treatment of NS by astragaloside should not replace the use of hormones, which is consistent with clinical practice [23].

Immunosuppression therapy is the most important treatment for the NS. However, many patients may relapse or resistant after the therapy [24]. Prolonged or repeated steroid therapy can lead to a variety of serious side effects [24]. Antibiotics may be needed to control infections. Angiotensin converting enzyme inhibitors, and diuretic medications are used to treat NS. Though many of these drugs are effective on the treatment of NS, they also cause many adverse effects including infection, osteoporosis,
suppression of bone marrow and liver damage\textsuperscript{25-26}. In China, traditional Chinese herbal medicines are commonly used in the treatment of NS\textsuperscript{11,26-27}. Most of the physicians consider them could increase the remission rate and reduce the adverse effect\textsuperscript{26}. Studies have reported that Astragaloside is safe and well tolerated, and the adverse events, such as raised total bilirubin and rash, are mild and resolved spontaneously\textsuperscript{28}. Our studies showed that the levels of serum CHO and TG in the astragaloside group were lower than that in the model group. TG was lower in the astragaloside group than in the MP group, suggesting that the regulation of high TG in rats with adriamycin nephropathy by astragaloside was better than that by hormones. These effects are favorable for preventing further kidney injury caused by hyperlipidaemia. By studying the expression of podocin and nephrin in rats with adriamycin nephropathy, nephrin and podocin were confirmed to be important for the maintenance of the structural integrity and normal function of the glomerular filtration barrier. Astragaloside improved the expression of the glomerular podocyte-related proteins nephrin and podocin in rats with adriamycin nephropathy, thereby improving glomerular podocytes, maintaining SD integrity, reducing proteinuria in NS, increasing blood ALB, and regulating blood lipids. The present study provided a basis for using astragaloside in the clinical treatment of NS. However, the effects of astragaloside on podocytes and of signaling transduction pathways and the internal regulatory mechanism require further study.

References


**Cite this article as:** WANG Na, WEI Ri-Bao, LI Qing-Ping, YANG Xi, CHEN Xiang-Mei. Protective effects of astragaloside in rats with adriamycin nephropathy and underlying mechanism [J]. *Chin J Nat Med*, 2016, 14(4): 270-277