

EFFECT OF SHEN-QI-DI-HUANG DECOCTION ON REDUCING PROTEINURIA BY PRESERVING NEPHRIN IN ADRIAMYCIN-INDUCED NEPHROPATHY RATS

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Abstract

The aim of this study is to investigate the effect of Shen-qi-di-huang decoction on reducing proteinuria and to discuss the mechanism of its action in Adriamycin (ADR)-induced nephropathy rats. The rats were randomly divided into three groups (n=12 each group): normal control (group A); ADR model control (group B); ADR + Shen-qi-di-huang decoction (group C). In group B and C, the rats were intravenously injected with ADR (6.5mg/kg). The rats in group C were orally administrated with Shen-qi-di-huang decoction after the injection of ADR. On day 7, 14, 28, 56 after ADR injection, 24h urine protein was detected. On day 28, 56 after ADR injection, ALB, ALT, serum creatinine (Scr) and BUN were examined. The morphological changes of the kidneys were observed by light microscope and electron microscope on day 28, 56 after ADR injection. The expression of nephrin was determined by immunohistochemistry and RT-PCR on day 28, 56 after ADR injection. Compared with group B, 24h urine protein and Scr decreased in group C on day 56 (P<0.05). The expression of nephrin determined by immunohistochemistry and RT-PCR increased in group C on day 28, 56 (P<0.05). The morphology observed by light microscope and electron microscope improved in group C on day 28, 56. Shen-qi-di-huang decoction decreases proteinuria, protects kidney function, and ameliorates histopathology in ADR-induced rats by preserving nephrin expression.

Keywords: Shen-qi-di-huang decoction, proteinuria, nephrin, adriamycin nephropathy

Introduction

Proteinuria is a clinical signature of podocyte injury resulting in many renal diseases. The slit diaphragm (SD) which connects neighboring foot processes of podocytes represents a critical structure of protein filtration (Tarabra et al., 2009). One of the major components in SD is nephrin. An alteration in its expression will lead to proteinuria (Lowik et al., 2009). Therefore, it is essential to carry out a further research on nephrin.

Shen-qi-di-huang decoction is a well-known decoction in traditional Chinese medicine and it has been widely applied in the treatment of renal diseases for decreasing proteinuria from clinical observation (Holthofer, 2007). However, the mechanism of its action is still unclear. In order to unveil the mystery, experiments in multi levels are necessary.

The rat model of adriamycin (ADR)-induced nephropathy has been proved to be a very reproducible model of proteinuria. After administration of ADR, rats present proteinuria and progressive renal disease (Pippin et al., 2009).

The aim of our study is to investigate the effect of Shen-qi-di-huang decoction on reducing proteinuria and to discuss the mechanism of its action in ADR-induced nephropathy rats.

Materials and methods

Preparation of Shen-qi-di-huang decoction

The dose of each herb in Shen-qi-di-huang decoction for each rat per day was as follow: DangShen[Codonopsis

pilosula (Franch.) Nannf.] 0.20g, Huangqi [Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao] 0.41g, Shengdihuang [Rehmannia glutinosa Libosch.] 0.31g, Mudanpi [Paeonia suffruticosa Andr.] 0.20g, Zexie [Alisma orientalis (Sam.) Juzep.] 0.20g, Shanyao [Dioscorea opposita Thunb.] 0.31g, Shanzhuyu [Cornus officinalis Sieb. et Zucc.] 0.12g, Fuling [Poria cocos (Schw.) Wolf] 0.20g. It was based on the formula of rat's Body Surface Area ($A=K*W^{2/3}$, $K=9.1$, $W=220 \pm 20$ g). These herbs were purchased from the pharmacy of Hangzhou Hospital of Traditional Chinese Medicine, Hangzhou, China. The crude drugs were mixed in water and concentrated into about 80ml and kept at 4°C. Each rat was administrated with this decoction (2ml qd).

Experimental model and treatment protocol

Male Sprague-Dawley rats weighing 220 ± 20 g were obtained from Experimental Animal Center, Zhejiang Chinese Medical University, Hangzhou, China. The experiment was performed according to the guidelines set by the World Health Organization. All rats lived in a temperature-controlled room (22°C) with a 12:12-h light-dark cycle and had free access to food and water. The rats were randomly divided into three groups (n=12 each group): normal control (group A); ADR model control (group B); ADR + Shen-qi-di-huang decoction (group C). In group B and C, the rats were injected with ADR (6.5mg/kg) diluted in 0.9% saline via tail vein (Zhejiang Hisun Pharmaceutical Co. Ltd. Taizhou, China). While in group A, the rats were injected with 0.9% saline instead. Each rat in group C was given Shen-qi-di-huang decoction through a gastric tube (2ml qd) after the injection of ADR. The rats in groups A and B were administrated with the same dose of water.

Collection of specimens

The 24h urine samples were collected by metabolic cages on day 7, 14, 28, 56 after ADR injection. The 24h urine protein was detected by Coomassie Brilliant Blue Method. The blood samples from the abdominal aortas on day 28, 56 were detected by Automatic Analyzer (7180, Hitachi, Tokyo, Japan).

Histologic analysis

Some of the kidneys were fixed in 10% formalin and embedded in paraffin for light microscopy and immunohistochemistry (BX51, Olympus, Tokyo, Japan). Some of them were fixed in 2.5% glutaraldehyde for electron microscopy (H600A, Hitachi, Tokyo, Japan). The rest were frozen in liquid nitrogen and stored at -80°C for gene analysis.

Immunohistochemistry

The paraffin sections were covered with anti-rat nephrin as primary antibody (1:20) (Santa Cruz Biotechnology, Inc. Santa Cruz, U.S.A) overnight at 4°C. Negative controls were covered with PBS rather than the primary antibody. Then the sections were incubated with EnVision+ /HRP/Rb (Gene Tech Co., Ltd, Shanghai, China) for 30 min at 37°C and dyed with 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China) under light microscope. The staining of nephrin was measured by Image-Pro Plus 6.0.

RT-PCR

There were three major steps of RT-PCR: the isolation of total RNA by TRIzol (CoWin Biotech Co., Ltd, Beijing, China), the reverse transcription by HiFi-MMLV cDNA Kit (CoWin Biotech Co., Ltd, Beijing, China) (37°C×40min, 70°C×10min) and the amplification as final step. The sequences of the primers were as follows (CoWin

Biotech Co., Ltd, Beijing, China):

nephrin:forwardprimer5'-GTCTGACGCTTTTGGCTTTC-3',reverseprimer5'-CTACCCAGGTCCTGTGCTT-3';

GAPDH:forwardprimer5'-CGTATCGGACGCCTGGTT-3',reverseprimer5'-GCTGACAATCTTGAGGGAGTTG-3'.

The optimum reaction conditions were 94°C×30sec, 60°C×50sec, 72°C×30sec, 32cycles, 72°C×5min. The image was captured by Gel Doc quantitative analysis system (Gel Doc 2000, Bio-Rad Laboratories, Hercules, USA) and analyzed by Quantity One.

Statistical analysis

Data were expressed as the mean ± SD. The measurement of data of multiple groups were conducted with one-way ANOVA (normal distribution) or rank sum test (non-normal distribution). The categorical variable data were conducted with Chi-square test. Correlation analysis was performed with linear correlation (normal distribution) or Spearman's rank (non-normal distribution).

Results

Urine and blood biochemical parameters

On day 7 after injection, ADR-induced rats showed a marked increase in 24-h urinary protein compared with group A ($P<0.05$). What's more, 24-h urinary protein of ADR- induced rats kept increasing until the end of this study. On day 7, 14, 28 after ADR injection, 24-h urinary protein in group C was lower than that in group B, but this was not statistically significant. On day 56, statistical difference was found between group B and group C ($P<0.05$) (Fig.1A).

On day 28, 56, ALB in group B and group C was lower than that in group A ($P<0.05$), but there was no significant difference in ALB between group B and group C (Fig.1B). On day 28, 56, there was no statistical difference in ALT (Fig.1C), BUN (Fig.1D) among the three groups. On day 28, the difference in serum creatinine (Scr) among three groups had no statistical significance. But on day 56, Scr in group B and group C was much higher than that in group A ($P<0.05$). In addition, Scr in group C was lower than that in group B, and there was a statistical difference between this two groups ($P<0.05$) (Fig.1E).

Morphological change in light microscope and electron microscope

Under the light microscope, glomerular morphology was grossly normal in group B and group C on day 28. Group B showed focal-segmental glomerulosclerosis, together with arteriosclerosis, inflammatory cell infiltration, interstitial fibrosis, and tubularcast formation on day 56. Group C had a certain improvement at the same time point (.Fig.2).

Under the electron microscope, the foot processes were mostly fused in group B on day 28. Rats in group B showed diffuse effacement of foot processes on day 56. Group C's condition was much better than group B at the same time point (Fig.3).

The pathological change of kidney under electron microscope and light microscopy resembled the human minimal change disease (MCD) on day 28. And the change was similar to human focal segmental glomerulosclerosis (FSGS) on day 56.

Nephrin immunohistochemistry and RT-PCR

The pattern of nephrin immunohistochemistry: The staining of nephrin was found continuously along the capillary loops in group A without expression in the tubules and interstitium. In group B, the positive staining of nephrin was

significantly attenuated and the distribution of staining shifted from a linear-like pattern to a coarse granule. In group C, the staining of nephrin increased. The result of Semiquantitative analysis (nephrin-positive area /glomerulus area *100%): On day 28, 56, the percentage of nephrin staining in group A was the highest among three groups, while in group B was the lowest ($P<0.05$). And it was significantly higher in group C than in group B at the same time point ($P<0.05$) (Fig.4).

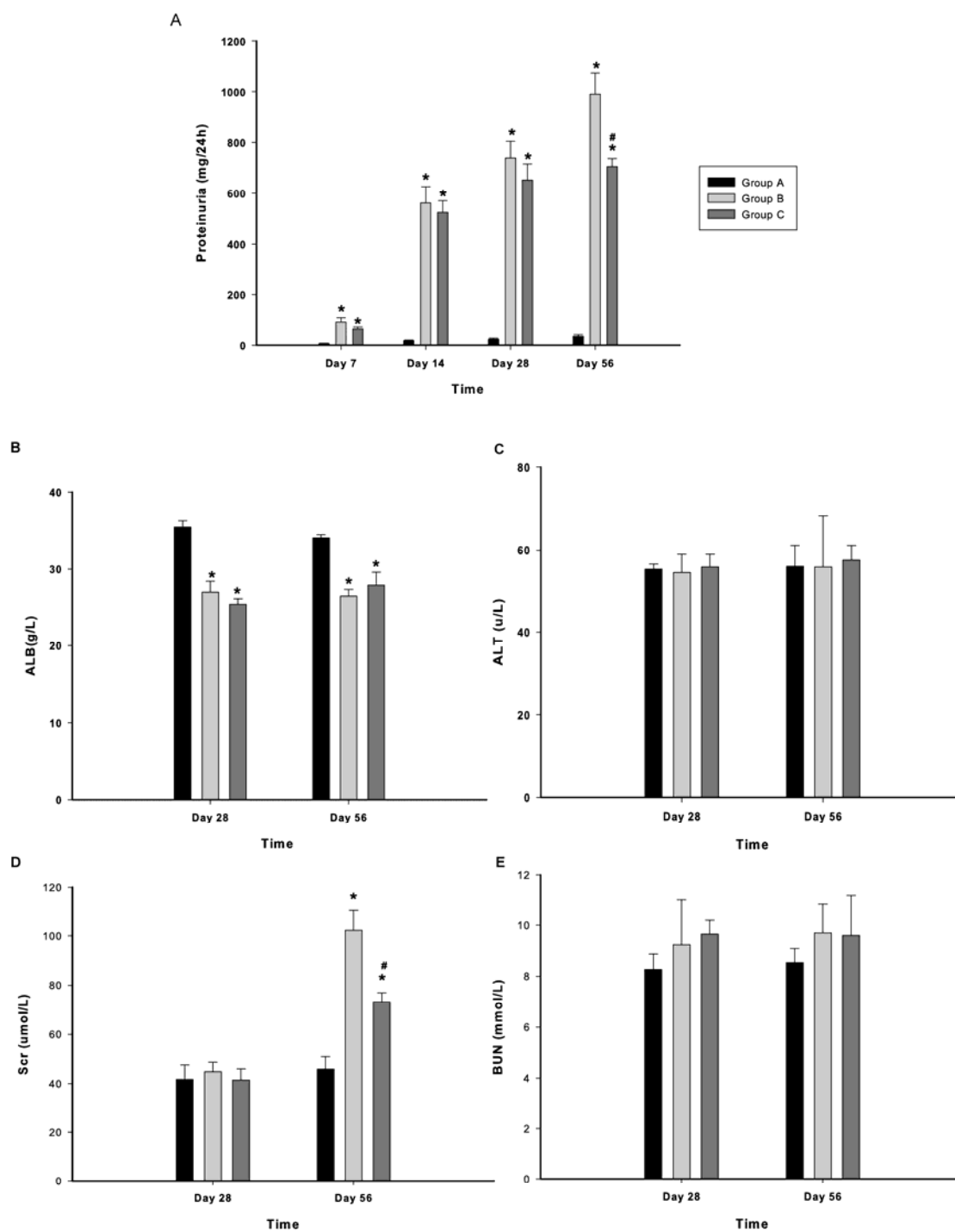


Figure 1: 24-h urinary protein(A), ALB(B), ALT(C), Scr(D), BUN(E) in rats of three groups on different time point. Normal control (group A); ADR model control (group B); ADR + Shen-qi-di-huang decoction (group C). * $P<0.05$ vs. group

A, # $P < 0.05$ vs. group B. (A: on day 7, 14, $n = 12$ respectively; on day 28, 56, $n = 6$ respectively) (B, C, D, E: on day 28, 56, $n = 6$ respectively)

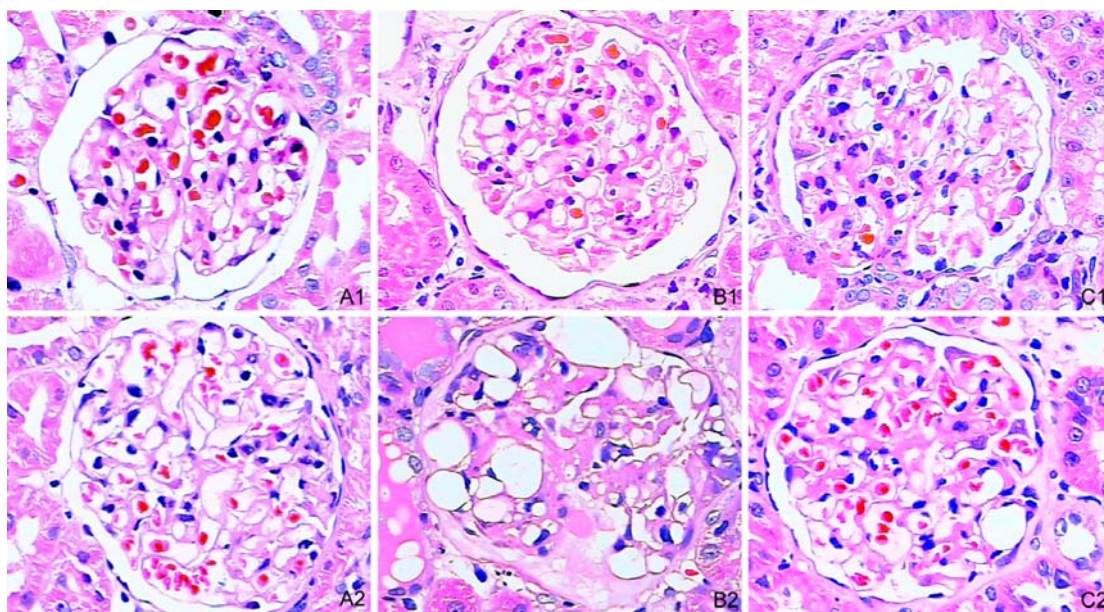


Figure 2: Light microscopic findings in rats of three groups (H.E. 400 \times). Normal control (group A); ADR model control (group B); ADR + Shen-qi-di-huang decoction (group C). (A1: group A on day 28, B1: group B on day 28, C1: group C on day 28, A2: group A on day 56, B2: group B on day 56, C2: group C on day 56)

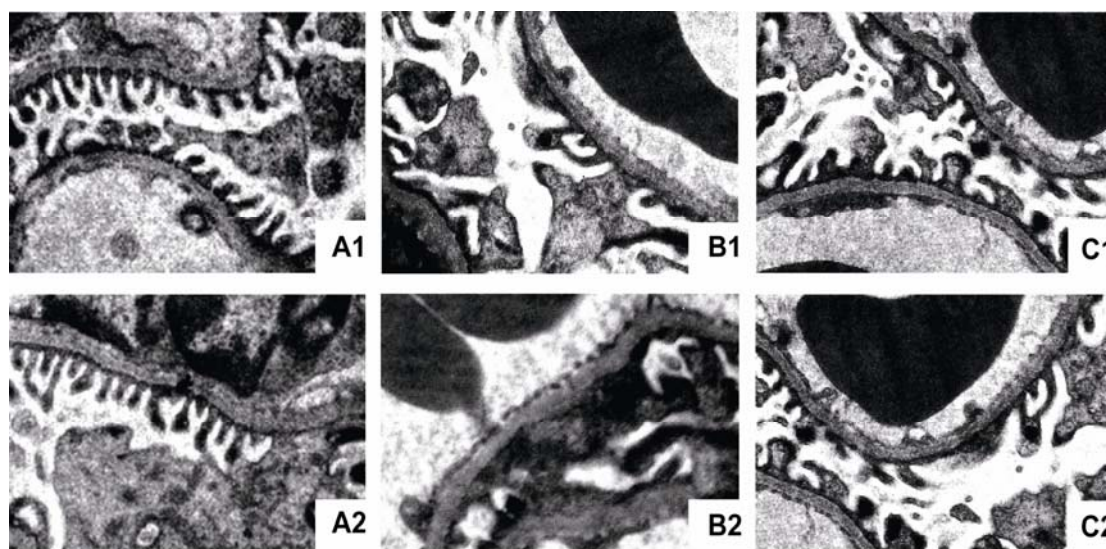


Figure 3: Transmission electron microscopic findings in rats of three groups (4000 \times). Normal control (group A); ADR model control (group B); ADR + Shen-qi-di-huang decoction (group C). (A1: group A on day 28, B1: group B on day 28, C1: group C on day 28, A2: group A on day 56, B2: group B on day 56, C2: group C on day 56)

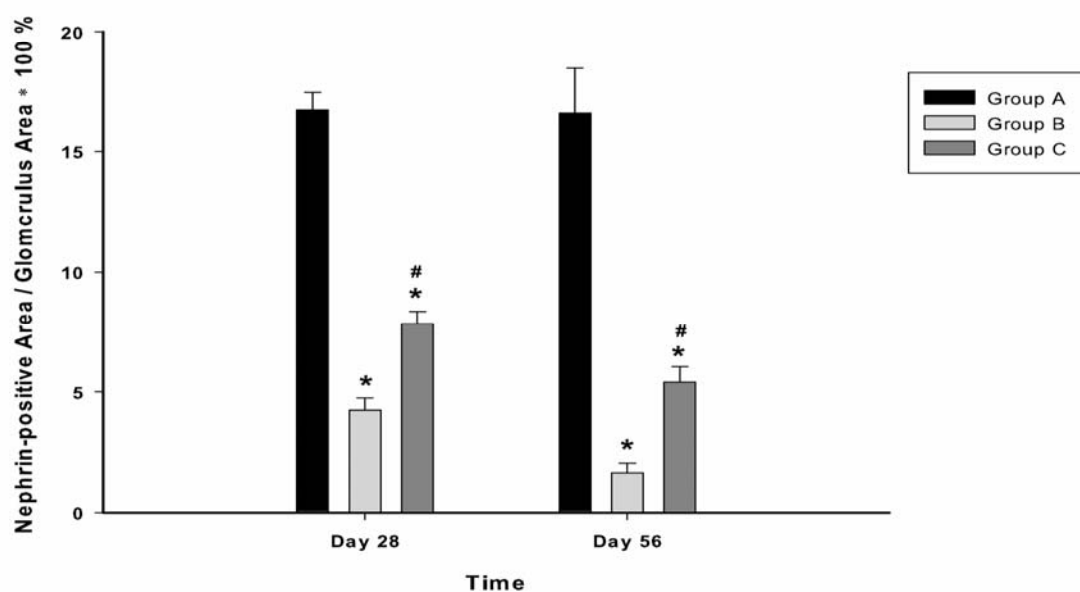
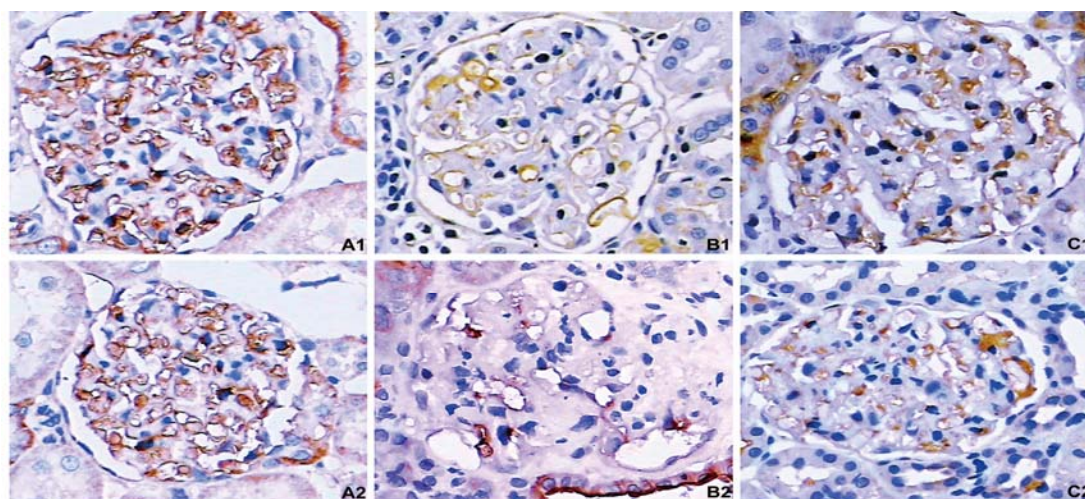


Figure 4: Immunostaining of nephrin in rats of three groups (400 \times). Normal control (group A); ADR model control (group B); ADR + Shen-qi-di-huang decoction (group C).n=6 respectively. * P <0.05 vs. group A, # P <0.05 vs. group B. (A1: group A on day 28, B1: group B on day 28, C1: group C on day 28, A2: group A on day 56, B2: group B on day 56, C2: group C on day 56)

The result of RT-PCR was consistent with the result of immunohistochemistry. The expression of nephrin mRNA in group B was down-regulated, compared to that in group A (P <0.05). However, the expression in group C was more than that in group B at the same time point (P <0.05) (Fig.5).

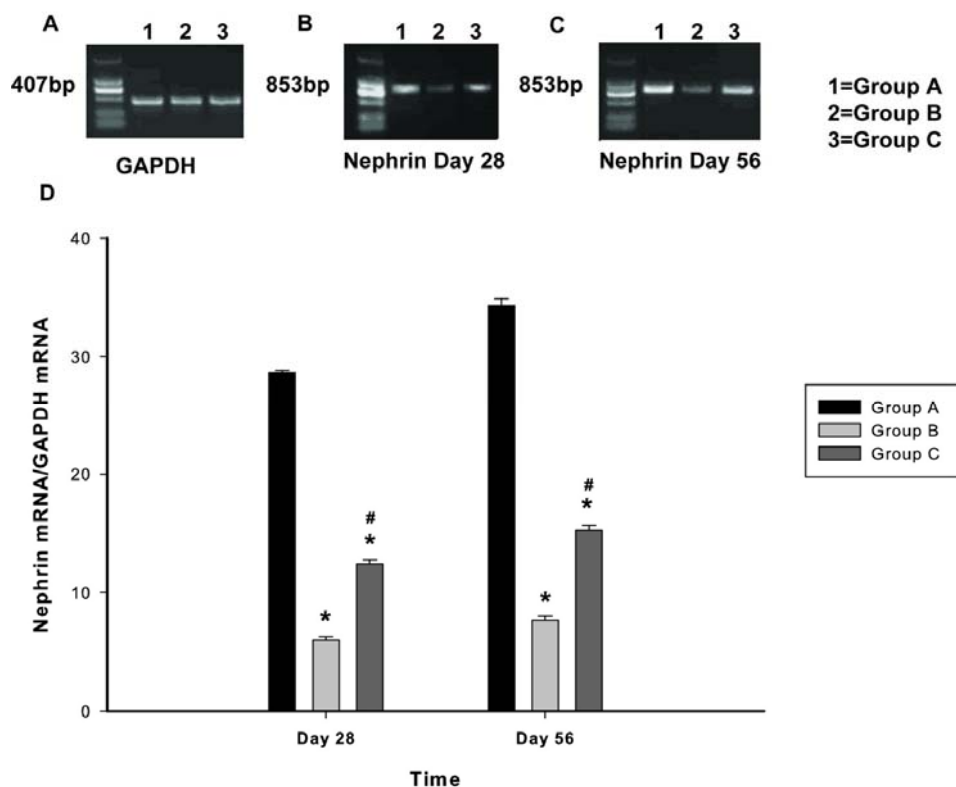


Figure 5: mRNA of nephrin in rats of three groups. Normal control (group A); ADR model control (group B); ADR + Shen-qi-di-huang decoction (group C).n=6 respectively. * $P < 0.05$ vs. group A, # $P < 0.05$ vs. group B. (A:GAPDH, B:nephrin day 28, C:nephrin day56, D: nephrin mRNA/ GAPDH mRNA in three groups on day 28,56)

Correlation analysis

The result of correlation Analysis showed that 24-h urinary protein was negatively correlated with the expression of nephrin both in immunohistochemistry ($r = -0.72$, $P < 0.05$) and RT-PCR ($r = -0.79$, $P < 0.05$) (Fig.6).

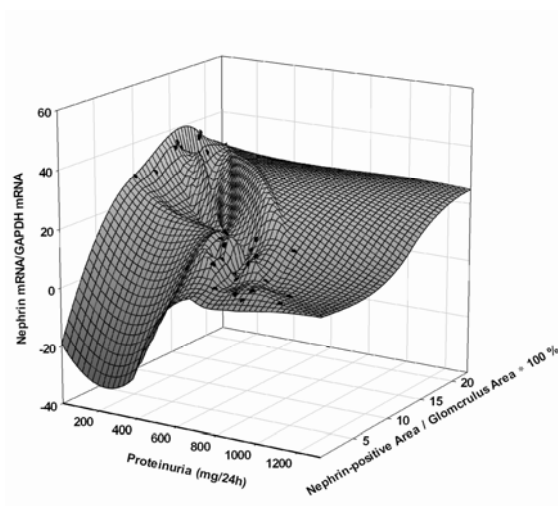


Figure 6: The relationship of 24-h urinary protein, nephrin mRNA/GAPDH mRNA and nephrin-positive area /glomerulus area *100%.

Discussion

It is known that ADR belongs to the anthracycline antibiotics. ADR-induced nephropathy has been recognized as a suitable model of podocyte disease. The pathophysiological mechanisms may be the initial cytotoxicity and the generation of reactive oxygen species (Gewirtz, 1999). The onset of proteinuria within 5 to 7 days after ADR injection serves as a good measure of adequate disease induction. Due to different administrations and doses of ADR, rats present different renal diseases on different time points: podocyte foot process fusion and effacement by week 4 to 8, segmental sclerosis by week 4 to 16, global sclerosis and interstitial fibrosis by week 6 to 24, death of uremia of some rats by week 28 (Pippin et al., 2009). In the present study, we successfully made the rat model of ADR-induced nephropathy by a single injection of 6.5mg/kg ADR via tail vein. According to the result of urine and blood biochemistry, kidney function becomes impaired with ADR, but there is not enough evidence to support the liver injury. In addition, the histopathological change from MCD to FSGS demonstrates the progression of the disease. It is a pity that the experiment stopped on day 56 after ADR injection, or we would have had an opportunity to observe the following progress of the diseases. But one point is clearly made that even induction with a single dose of ADR, rats would develop persistent nephropathy.

Proteinuria is an early indicator of varied renal diseases and a risk factor of renal impairment, hypertension, and cardiovascular disease. Moreover, recent epidemiologic data suggests that proteinuria is a more important determinant than the glomerular filtration rate (Danziger, 2008). Recently more and more attentions are paid to the SD, as it is the vital barrier to prevent circulating proteins into urine. Nephritin is the first demonstration of a known protein located in the SD. It has been known that nephritin is a transmembrane protein of the immunoglobulin superfamily encoded by NPHS1 and it acts as a 'signaling node' in the SD by transmitting extracellular signals from the SD to the intracellular actin cytoskeleton (Chuang et al., 2009). The massive observations suggest that nephritin is involved in the development of proteinuria not only in congenital nephrotic syndrome of the Finnish-type, but also in acquired glomerular diseases (Kawachi et al., 2002). Some studies demonstrate that lack of nephritin results in a loss of the SD, foot process dysmorphology and massive proteinuria (Putaalaa et al., 2001). While some studies indicate that the expression of nephritin is up-regulated or unchanged in kidney diseases (Aaltonen et al., 2001; Koop et al., 2003; Schaefer et al., 2004), our result supports the former. In our study, the proteinuria was negatively correlated with the expression of nephritin in ADR-induced rats observed by immunohistochemical staining and RT-PCR, which lasted until the end of this experiment. What's more, the severe disruption of SD structure observed by light microscope and electron microscope conformed to the lower level of nephritin expression. Loss of nephritin expression may derive from reduced protein synthesis or exaggerated protein degradation. It is likely that decreased nephritin expression may predispose podocytes to a variety of injury. Our study suggests that nephritin is of importance to maintain the integrity of the glomerular filtration barrier and prevent proteinuria.

Shen-qi-di-huang decoction is an effective prescription of Chinese herbal medicine for varied chronic diseases with Qi and Yin deficiency of liver, spleen and kidney, especially for renal diseases. This decoction is composed of liuwei dihuang decoction (Shengdi, Danpi, Zexie, Shanyao, Shanzhuyu, Fuling), dangshen and huangqi. Some researches indicate Liuwei dihuang decoction restores the metabolite network (Xie et al., 2009) and increases insulin sensitivity (Wu et al., 2002). Dangshen mediates inflammatory responses with GM-CSF (Byeon et al., 2009) and up-regulates antioxidant enzymes, catalase and superoxide dismutase (Tsai et al., 2008). Huangqi induces apoptosis selectively in cancer cells and has the effects of anti-inflammatory, antiviral, antioxidant (Liang et al., 2010). In the present study, treatment with Shen-qi-di-huang decoction decreased proteinuria in ADR-induced rats to a certain extent and improved the kidney function as well. Moreover, Shen-qi-di-huang decoction preserved the expression of nephritin in ADR-induced rats by immunohistochemistry and RT-PCR. Furthermore, loss of podocytes and fusion of foot processes in ADR-induced rats were prevented by the treatment of Shen-qi-di-huang decoction. Partial preservation of nephritin may shed light on why Shen-qi-di-huang decoction plays an important role in treating chronic kidney diseases.

Conclusion

In conclusion, the primary achievement in our study is Shen-qi-di-huang decoction decreases proteinuria, protects kidney function, and ameliorates histopathology in ADR-induced rats by preserving nephrin expression. But there are still some limitations in our experiment. Further studies are needed to examine whether other proteins, like podocin, are affected by Shen-qi-di-huang decoction. The combination of Shen-qi-di-huang decoction and western medicine also need to be investigated in future.

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