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EXPERIMENTAL STUDY

Artemisinin ameliorated proteinuria in rats with adriamycin-induced nephropathy through regulating nephrin and podocin expressions

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Abstract

OBJECTIVE: To investigate the effects of artemisinin against proteinuria and glomerular filtration barrier damage in rats with adriamycin-induced nephropathy, and the potential mechanism underpinned the action.

METHODS: Forty adriamycin rats were randomly divided into two groups with the ratio of 1 : 3; the small-number group served as control group (n=10), and the rats in the large-number group were treated with adriamycin to induce nephropathy; then they were further randomly assigned into 3 subgroups: benazepril group (n=10), artemisinin group (n=10), and adriamycin group (n=10). The benazepril group and artemisinin group were treated with benazepril suspl (5.0 mg/kg daily) and artemisinin suspl (150 mg/kg daily) respectively after being modeled; those in the control group and adriamycin group were intragastrically administered an equivalent volume of distilled water every day. The treatment after model establishment lasted for a total of 4 weeks. The 24 h uric protein, blood biochemicals, renal pathological changes, renal ultrastrutural changes, Nephrin and Podocin proteins and gene expressions were measured by Coomassie brilliant blue assay, completely automatic biochemical analyzer, light microscope, electron microscopy, Western blot and reverse transcription polymerase chain reaction, respectively.

RESULTS: The rats in adriamycin group showed a significant increase in 24 h uric protein excretion, serum total cholesterol (TC), triglyceride (TG), blood urea nitrogen (BUN), serum creatinine (Scr) and decrease in albumin (Alb) (P<0.05 or P<0.01). Compared with adriamycin group, artemisinin could reduce uric protein excretion, decrease the serum TC, TG elevation, increase the serum Alb level, up-regulate the expressions of Nephrin and Podocin (P<0.05 or P<0.01), but no statistical significance effects on the levels of BUN, Scr in artemisinin group (P>0.05). The renal pathological and ultrastrutural observation indicate that artemisinin could attenuate the severity of foot process effacement and fusion in the nephropathic rats.

CONCLUSION: Artemisinin might have an effect on the nephropathy in rats caused by adriamycin, which may be at least partly correlated with attenu-

ation of the severity of foot process effacement and fusion, up-regulation of the expressions of Nephrin and Podocin in the glomeruli in the rats.

Key words: Artemisinin; Proteinuria; Doxorubicin; Kidney diseases; Nephrin; NPHS2 protein

INTRODUCTION

Proteinuria is an important risk factor for the progression and prognosis of chronic kidney disease (CKD),[1] including minimal change nephropathy, focal segmental glomerular sclerosis, mesangial proliferative glomerulonephritis, membranous nephropathy, diabetes mellitus, and lupus nephritis.[2,3] Thus, the decreased urine protein excretion level is associated with a slower deterioration in renal function. Podocytes, a kind of highly differentiated cells, forming multiple interdigitating foot processes, are interconnected by the slit diaphragms (SD)[4] and implicated in the development of proteinuria.[5] The genes that encode the podocyte-associated proteins Nephrin and Podocin help maintain the integrity of SD and prevent proteinuria.[6] This suggests that Nephrin and Podocin play important roles in maintaining the structural and functional integrity of the glomerular filtration barrier and the development of proteinuria. At present, its treatment is more dependent on glucocorticoid, and cytotoxicand neotype immunodepressive drugs etc. However, glucocorticoid resistance and dependence, and their side effects are formidable problems. Therefore, novel therapeutic strategies are necessary for a better clinical management of the nephritic syndrome.

Artemisinin is a new type of sesquiterpene lactones compound contained hydroperoxyradical group which was extracted from artemisia annua L and A • apiacea Hance by pharmaceutical workers of our country in 1971. It has the functions of immunological regulation, anti-fibrosis, cell proliferation inhibition and apoptosis inducement.[7-10] In previous studies, we found that artemisinin could reduce proteinuria/24 h, decrease the levels of TNF-α, IL-6 and down-regulate the expression of the NF-kBp65 protein and NF-kB, TGF-β1 mRNA in renal tissues.[11] Theoretically, it is possible that artemisinin could be used to treat other kidney diseases. However, the therapeutic effect and mechanisms are still unclear. The aims of the present study are to investigate its protective effects on adriamycin-induced nephropathy in rats' models, and to discuss the possible mechanism underlying the action.

MATERIALS AND METHODS

Animal

Forty healthy Male Sprague-Dawley rats of SPF grade, eight-week-old, weighing (200 ± 20) g were purchased from the Experimental Animal Center of Medical School of Xi'an Jiaotong University (Certificate of quality No. 2008016). The experimental procedures were approved by the Animal Ethics Committee of Medical School of Xi'an Jiaotong University (Xi'an, China). All animals were maintained in individual metabolic cages in an air-conditioned room at a constant temperature 23℃±2℃ with a 12 h light/dark cycle, and allowed ad libitum access to water and standard pelleted diet containing 18% protein (w/w).

Drugs

Artemisinin (white to whitish needle crystalls or crystal powder, 99%) was purchased from Xi'an Natural Field Bio-technique Co., Ltd. (Xi'an, China), and benazepril hydrochloride tablets (10 mg per tablet) from Beijing Novartis Pharma Ltd. (Beijing, China).

Instruments and reagents

RA-1000 completely automatic biochemical analyser was purchased from Amresco (Boston, MA, USA); CX31 light microscope and HITACHI-600 transmission electron microscope was purchased from Nikon (Tokyo, Japan); Q500IW image analysis system was purchased from Leica (Dresden, Germany); Coomassie brilliant blue assay was purchased from Amresco (Boston, MA, USA); TRIZOL reagent was purchased from Invitrogen (Carlsbad, NM, USA); Lowry method (DC Protein Assay, Biorad, Hercules, CA, USA).

Grouping, modeling and processing methods of the animal

After 1 week of routine adaptive feed, all experiments rats were randomly divided into two groups with the ratio of 1∶3 by random number table method. Adriamycin (7.5 mg/kg) was singly administered through the tail vein of the rats in the large-number group (n=30) to establish rats' models. An equivalent volume of normal saline was given to the rest as blank control group (n=10). Seven days after the injection, 24 h urine samples were collected and the adriamycin-injected rats were randomly divided into three subgroups (10 for each) according to random number table. They were adriamycin group, benazepril group, and artemisinin group. The benazepril group and the artemisinin group were administrated with benazepril suspl (5.0 mg/kg daily) and artemisinin suspl (150 mg/kg daily) respectively. Rats in the blank control group and adriamycin group were intragastrically administered an equivalent volume of distilled water every day. The treatment lasted for a total of 4 weeks.

24 h urine samples were collected to measure urinary volume and urine protein level by using metabolic cages on the 1th, 2th, 3th, 4th week. All rats were sacrificed at the end of the experiment. The blood serum was collected. Their kidneys were harvested, weighed, and cut into portions for the examination with electron microscopy (EM) and light microscope, and for

the preparation of glomerular protein and RNA. Western blot and Semi quantitative RT-PCR were carried out on glomerular protein and mRNA for Nephrin and Podocin.

Experimental procedures

Measurement of uric protein: each animal was housed separately in a metabolic cage to measure the 24 h uric protein excretion, and the daily urine volume was measured. Uric protein was analyzed with Coomassie brilliant blue assay (Amresco, Boston, MA, USA).

Blood biochemical measurement: serum albumin (Alb), Triglyceride (TG), total cholesterol (TC), blood urea nitrogen (BUN) and serum creatinine (Scr) levels were measured with a RA-1000 completely automatic biochemical analyser (Boston, MA, USA).

Light microscope investigation: renal tissue samples were put into 10% formaldehyde for fixation. Three-micrometer thick paraffin histological sections of the kidney cortex were observed and imaged under light microscope (Pattern number: CX31, Nikon Company, Tokyo, Japan).

Transmission electron microscopy investigation: renal tissue samples for electron microscopic assessment were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (PB) for several days at 4℃. After washed in PB and post-fixing in 1% OsO_4 for 2 h, the fixed material was dehydrated through an ethanolpropylene oxide series and embedded in Araldite M. The ultrathin sections were prepared and stained with uranyl acetate and lead citrate, and then they were investigated and photographed under a HITACHI-600 transmission electron microscope (Tokyo, Japan).

Western blotting: the renal cortex and isolated glomeruli were homogenized in a Dounce homogenizer in RIPA buffer [consisting of 0.1% sodium dodecyl sulphate (SDS), 1% TritonX-100, 150 mM NaCl, 1% sodium deoxycholate and 10 mM ethylenediaminetetraacetic acid [EDTA] in 25 mM Tris-HCl, pH7.2] and the protein concentrations of these samples were assayed by the Lowry method (DC Protein Assay, Biorad, Hercules, Santa Cruz, CA, USA). For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), samples were mixed with an equal volume of sample buffer and boiled for 5 min. Proteins (10 mg/lane) were run on 10% polyacrylamide slab gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skimmed milk in PBS with 0.1% Tween 20 for 1 h at room temperature and incubated overnight with the primary antibodies (antibody concentrations were one-tenth of those used for IFM) at 4℃ . The membranes were then incubated with a HRP-labeled goat anti-rabbit IgG. The bound secondary antibody was detected by enhanced chemiluminescence. Housekeeping protein β-actin was used as a loading control. Positive immunoreactive bands were quantified densitometrically (Leica Q500IW image analysis system) and normalized for β-actin.

RNA extracting and RT-PCR: the renal cortex samples were flash-frozen in liquid nitrogen and stored at - 80℃. The total RNA (1 μg) extracted from renal cortex with TRIZOL (Invitrogen, Carlsbad, CA, USA) was reverse-transcribed using superscript reverse transcriptase to yield the respective cDNA. The efficiency of the RT-PCR was controlled by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification. Each sample mixture contained a standard PCR buffer, 10-mM dNTP, 2 U Taq polymerase, and 5 pM of each of the following primers: GAPDH, 5'-CAAGTTCAACGGCACAGTCAA-3'and 5'-TGGTGAAGACGCCAGTAGACTC-3'; Nephrin, 5'-GGCGTAGCTTAGGGAC-3' and 5'-CCTAGCCGCCAATCAC-3'; Podocin, 5'-CTAAGCAG TCTAGCTCATG-3' and 5'-CAATCACCCGCACTTT-3'; The PCR program consisted of the following steps: 95℃ for 5 min; a denaturation step at 94℃ for 30 s; an annealing step at 52.3℃ (GAPDH), at 53.1℃ (Nephrin), at 54.1℃ (Podocin); and an extension step at 72℃ for 30 s, then 36 cycles for Nephrin and Podocin; 28 cycles for GAPDH; and a final extension at 72℃ for 7 min. The expected PCR product size was 596 bp for GAPDH, 299 bp for Nephrin, 392 bp for Podocin. The PCR products were subjected to a computer-assisted densitometry after electrophoresis on a 1% agarose gel and staining with ethidium bromide.

Statistical analysis

The data were analyzed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The differences among the four groups were tested using a one-way ANOVA with SNK-*q* test. All values are expressed as the mean ± *SD*, and $P<0.05$ was considered statistically significant

RESULTS

24 h uric protein excretion

As shown in Table 1, during the observation period, the rats in adriamycin group showed a marked increase in 24 h uric protein excretion from the 1th week of the experiment. After 4 weeks treatment, 24 h uric protein excretion in the artemisinin and benazepril group was less than that of the Adiamycin group, and there was no significant difference between artemisinin group and benazepril group.

Blood biochemicals change

As shown in Table 2, during the observation period, the rats in adriamycin group showed a significant increase in blood TG, TC, BUN, Scr and decrease in Alb. After 4 weeks treatment, the serum levels of TG and TC were lower and the serum Alb levels higher in the benzaepril and arteminsin groups compared to the adriamycin group, but there was no obviously change in BUN and Scr levels in benzaepril and arteminsin groups, and no significant difference between artemisinin group and benazepril group.

Table 1 24 h uric protein excretion (mg/24 h, $\bar{x} \pm s$)

Group	*n*	1th week	2th week	3th week	4th week
Blank control	10	8.78±1.87[a]	8.78±1.87[a]	9.01±1.32[a]	8.56±1.35[a]
Adriamycin	10	62.02±11.22	66.33±13.89	90.12±15.79	87.76±18.66
Benazepril	10	52.55±12.87[a]	57.23±13.11[a]	75.55±17.26[a]	77.54±15.22[a]
Artemisinin	10	53.09±11.01[a]	50.13±10.12[a]	72.32±11.99[a]	70.69±13.99[a]

Notes: blank control and adriamycin group were administrated only with equivalent volume of distilled water every day; benazepril group were administrated with benazepril suspl (5.0 mg/kg daily); artemisinin group were administrated with artemisinin suspl (150 mg/kg daily). [a]*P*<0.01, compared with the adriamycin group.

Table 2 Blood biochemicals change ($\bar{x} \pm s$)

Group	*n*	TG (mmol/L)	TC (mmol/L)	Alb (g/L)	BUN (mmol/L)	Scr (μmol/L)
Blank control	10	1.81±0.31[a]	1.43±0.35[a]	35.22±0.89[b]	5.42±0.58[a]	27.30±6.11[a]
Adriamycin	10	4.63±1.55	3.84±1.08	31.59±1.60	8.32±0.64	45.30±6.03
Benazepril	10	3.12±1.66	2.78±1.21[b]	32.99±1.97	7.98±0.57	43.22±5.79
Arteminsin	10	1.98±0.42[a]	2.11±0.67[b]	34.78±0.66[b]	7.76±0.61	40.60±6.01

Notes: blank control and adriamycin group were administrated only with equivalent volume of distilled water every day; benazepril group were administrated with benazepril suspl (5.0 mg/kg daily); artemisinin group were administrated with artemisinin suspl (150 mg/kg daily). Alb: albumin; TG: triglyceride; TC: total cholesterol; BUN: blood urea nitrogen; Scr: serum creatinine. [a]*P*<0.01, [b]*P*<0.05, compared with the adriamycin group.

Renal pathological changes

As shown in Figure 1, the rats in control group were found to have normal glomerular structures, opened glomerular capillary lumens, a smaller width of the mesangial region than that of the blood vessel diameter, and normal renal tubule and interstitial substance. The typical pathological manifestation of the rats in adriamycin group was glomerular atrophy in part of the glomeruli. This was seen mainly as narrowing of the capillary lumen, varying degrees of asymmetric mesangial proliferation, and enlargement of the mesangial region (the width of glomerular mesangial region is larger than the diameter of the capillary). The rats in benazepril group and artemisinin group had a smaller width of the glomerular mesangial region and a relatively wider.

Renal ultrastrutural changes

As shown in Figure 2, electron microscopy investigations showed no podocyte shape changes, such as edema cytoplasm and foot process effacement in control group (Figure 2A), there were foot process of GBM widened, recoveried, and part of them fused and efface-

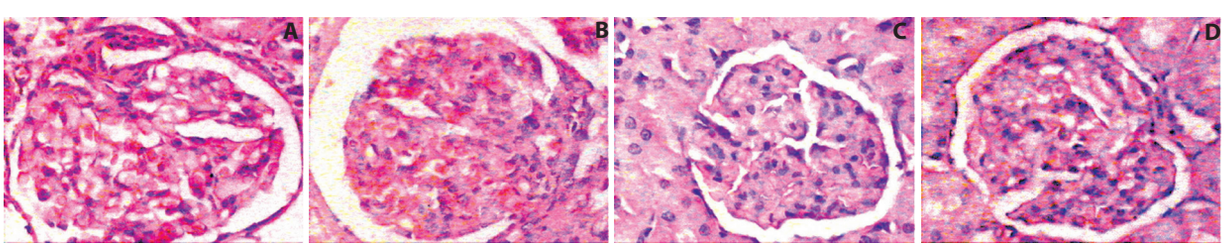

Figure 1 Renal pathological changes (HE staining, ×400)

A: blank control group; B: adriamycin group; C: benazepril group; D: artemisinin group. Blank control and adriamycin group were administrated only with equivalent volume of distilled water every day; benazepril group were administrated with benazepril suspl (5.0 mg/kg daily); artemisinin group were administrated with artemisinin suspl (150 mg/kg daily). HE: hematoxylin and eosin stain.

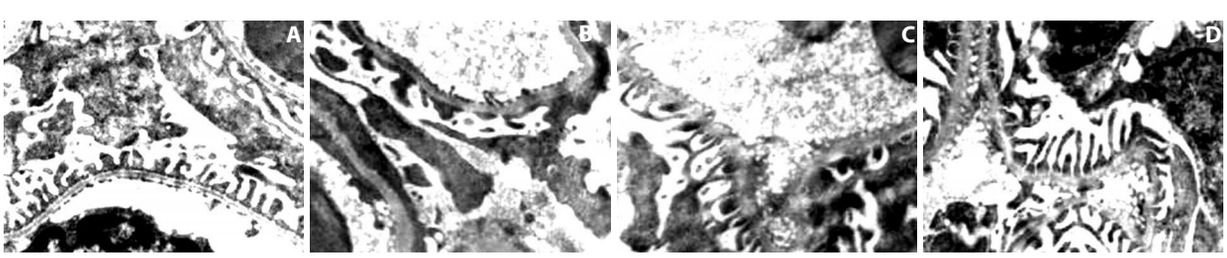

Figure 2 Renal ultrastrutural changes (transmission electron microscopy; original magnification: ×10 000)

A: blank control group; B: adriamycin group; C: benazepril group; D: artemisinin group. Blank control and adriamycin group were administrated only with equivalent volume of distilled water every day; benazepril group were administrated with benazepril suspl (5.0 mg/kg daily); artemisinin group were administrated with artemisinin suspl (150 mg/kg daily).

ment. After 4 weeks treatment, Edema cytoplasm was defluxioed and reduced obviously in adriamycin group (Figure 2B), the pathological changes were improved, and foot process structure was cleared in benazepril group and artemisinin group (Figure 2C and D).

Expressions of Nephrin and Podocin proteins

Western blot analyses were performed for assessing Nephrin and Podocin protein. As shown in Table 3 and Figure 3, the artemisinin treatment up-regulated the protein expressions of Nephrin and Podocin ($P<0.01$) in glomeruli, and there was no significant difference between the artemisinin group and benazepril group.

Table 3 Expressions of nephrin and podocin protein ($\bar{x} \pm s$)

Group	*n*	Expression of nephrin	Expression of podocin
Control	10	0.341±0.032[a]	0.363±0.029[a]
Adriamycin	10	0.126±0.028	0.123±0.025
Benazepril	10	0.242±0.039[a]	0.285±0.040[a]
Artemisinin	10	0.264±0.027[a]	0.291±0.039[a]

Notes: blank control and adriamycin group were administrated only with equivalent volume of distilled water every day; benazepril group were administrated with benazepril suspl (5.0 mg/kg daily); artemisinin group were administrated with artemisinin suspl (150 mg/kg daily). [a]$P<0.01$, compared with the adriamycin group.

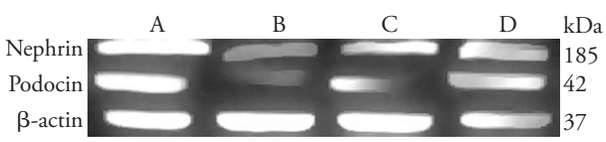

Figure 3 The expressions of nephrin and podocin at protein level

A: blank control group; B: adriamycin group; C: benazepril group; D: artemisinin group. Blank control and adriamycin group were administrated only with equivalent volume of distilled water every day; benazepril group were administrated with benazepril suspl (5.0 mg/kg daily); artemisinin group were administrated with artemisinin suspl (150 mg/kg daily).

Expressions of nephrin and podocin mRNA

Semi quantitative RT-PCR shown in Table 4 and Figure 4, the artemisinin treatment up-regulated the mRNA expressions of Nephrin and Podocin ($P<0.01$) in glomeruli, and there was no significant difference between the artemisinin group and benazepril group. These results indicate that artemisinin on the levels of mRNA expressions as the same effects as above the protein expressions.

Table 4 Expressions of nephrin and podocin mRNA (OD value, $\bar{x} \pm s$)

Group	*n*	Expression of nephrin mRNA	Expression of podocin mRNA
Control	10	1.18±0.12[a]	1.25±0.14[a]
Adriamycin	10	0.64±0.10	0.73±0.10
Benazepril	10	0.98±0.62[a]	1.00±0.66[a]
Artemisinin	10	1.12±0.61[a]	1.17±0.11[a]

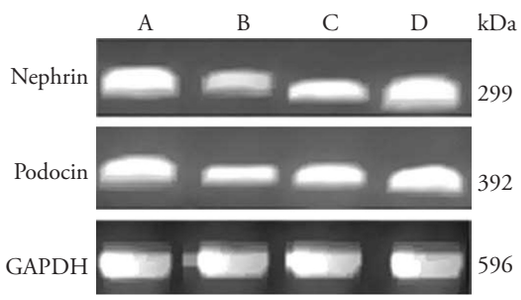

Figure 4 The expressions of nephrin and podocin at gene level

A: blank control group; B: adriamycin group; C: benazepril group; D: artemisinin group. Blank control and adriamycin group were administrated only with equivalent volume of distilled water every day; benazepril group were administrated with benazepril suspl (5.0 mg/kg daily); artemisinin group were administrated with artemisinin suspl (150 mg/kg daily). GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

DISCUSSION

Over the past decade, scientific research revealed that podocyte injury was involved in many forms of glomerular disease.[12] Podocytes are highly differentiated cells that have a limited ability to proliferate. A decrease in podocyte number has been shown to accompany massive proteinuria in many forms of glomerular disease. In addition, other researchers have found that a reduction in podocyte number results in the nudity of glomerular basement membrane (GBM) and, ultimately, in focal segmental glomerulosclerosis (FSGS).[13,14] This suggests that FSGS is triggered by a decrease in podocyte number to a certain extent.

Our study strived to address that artemisinin was able to alleviate proteinuria and attenuate the severity of foot process effacement and fusion in the adriamycin-induced nephropathy rats with comparable therapeutic efficacy to benazepril. Biochemically, compared with the rats in adriamycin group, the artemisinin treatment alone decreased the TC and TG levels and increased the serum Alb level in rats. These results indicate that artemisinin might have an effect on lipid metabolism.

Recent studies indicated that local podocyte damage could induce injury in otherwise healthy podocytes and further affect glomerular endothelial and mesangial cells. This implies that even limited podocyte injury can initiate a vicious cycle of progressive glomerular damage.[15] Podocytes are composed of three parts of different structure and function, including cell body, cardinal processes, and foot processes. There are slit pores among foot processes and the SD covering the slit pores near the GBM forms the outer layer of the glomerular filtration barrier. The SD also plays a key role

in protein filtration.[16] Nephrin is an important composes of the SD and is fundamental in keeping the glomerular filtration barrier function.[17] Lately, some experimental reports have shown that the anormal expression of Nephrin leads to massive proteinuria.[18,19] Podocin is a membrane-associated protein of the band-7- stomatin family that interacts with the cytosolic tail of Nephrin and connects Nephrin signalling to the cytoskeleton.[20] In the proteinuric state, down-regulation and shift localization of Podocin were observed.[21] Nakhoul *et al* [22] demonstrated in adriamycin rats that treatment with the angiotension converting enzyme inhibitor enalapril alone or in combination with losartan resulted in a significant preservation of Podocin. In the present study, we observed that both artemisinin and benazepril could modulate the protein and gene expressions of Nephrin and Podocin at the end of the experiment.

The present study demonstrated that artemisinin could decrease 24 h uric protein excretion and the serum levels of TG and TC, and increase the serum Alb level. The mechanism may be at least partly correlated with attenuation of the severity of foot process effacement and fusion, up-regulation of the protein and expressions of Nephrin and Podocin in the glomeruli from the modeled rats.

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