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Protective Effect of Sulodexide on Podocyte Injury in Adriamycin Nephropathy Rats*

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Summary：This study examined the effect of sulodexide on podocyte injury in rats with adriamycin nephropathy (AN). A total of 36 healthy male SD rats were randomly assigned to three groups: control group, AN group and sulodexide treatment group. Rat models of AN were established by a single tail intravenous injection of adriamycin (6.5 mg/kg) in both AN group and sulodexide treatment group. Sulodexide (10 mg/kg) was administered the rats in the treatment group once daily by garage from the first day of model establishment until the 14th day or the 28th day. Samples of 24-h urine and renal cortex tissues were harvested at day 14, 28 after the model establishment. Excretion of 24-h urinary protein was measured by Coomassie brilliant blue method. The pathological changes in renal tissues were observed by light microscopy and electron microscopy respectively. Heparanase mRNA was detected by RT-PCR. Expressions of desmin, CD2AP and heparanase were determined by immunohistological staining. The results showed that the expressions of heparanase mRNA and protein were increased in the glomeruli of AN rats at day 14 and 28 after the model establishment, which was accompanied by the increased expression of desmin and CD2AP. The mRNA and protein expression of heparanase was decreased in the sulodexide-treated rats as compared with AN rats at day 14 and 28. And, the protein expression of desmin and CD2AP was reduced as with heparanase in the sulodexide-treated rats. Proteinuria and podocyte foot process effacement were alleviated in the AN rats after sulodexide treatment. There was a positive correlation between the expression of heparanase and the expression of desmin and CD2AP (as well as 24-h urinary protein excretion). It was concluded that increased heparanase is involved in podocyte injury. Sulodexide can maintain and restore podocyte morphology by inhibiting the expression of heparanase in AN.
Key words：sulodexide; podocyte; heparanase; adriamycin nephropathy

Many chronic kidney diseases are characterized by proteinuria which leads to renal fibrosis and sclerosis, and accelerates the progression to end-stage renal disease (ESRD). Podocyte is a kind of terminally differentiated epithelial cell that lines the outer aspect of the glomerular basement membrane (GBM). From the cell body arise several primary processes, which give forth many foot processes. The glomerular slit diaphragm (SD) represents the junction structure that links the interdigitating foot processes from neighboring podocytes. Podocytes and the SD complex serve as the final barrier against protein leakage from the kidney[1]. Podocyte injury and SD damage are generally believed to be associated with massive proteinuria[2].

Recently, glycosaminoglycan(GAG)-based drugs have been used to treat proteinuria[3]. And, sulodexide, which is composed of 80% heparin and 20% dermatan sulfate, has been shown to effectively decrease urinary albumin excretion rates (AERs) in diabetic nephropathy[4, 5] and other glomerular diseases[6–8]. However, the underlying mechanism remains unclear.

In this study, the rat model of adriamycin nephropathy (AN) was established to examine the effect of sulodexide on adriamycin-induced podocyte injury and the possible mechanism.

1 MATERIALS AND METHODS

1.1 Model Establishment and Grouping

Thirty six healthy male SD rats, aged from 6 to 8 weeks and weighing from 160–200 g, were provided by the Experimental Animal Center of Tongji Medical College, HUST, China. The rats were randomly assigned to three groups: control group, AN group and sulodexide treatment group (sulodexide group). The rats in groups AN and sulodexide were given a single tail intravenous injection of adriamycin (6.5 mg/kg)[9]. Sulodexide (10 mg/kg)[10] was administered the rats in the sulodexide group once daily by gavage for 14 or 28 days after AN induction.

1.2 Determination of 24-h Urinary Protein Excretion

All the rats were placed in metabolism coops at day

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13 and 27. The samples of 24-h urine were collected at day 14 and 28. Excretion of 24-h urinary protein was determined by Coomassie brilliant blue method.

1.3 Preparation of Kidney Tissue Samples

Six rats from each group were sacrificed at day 14 and 28 after model establishment by giving a lethal dose of Nembutal. Kidney tissues were harvested. One fourth cortex tissue of left kidney was fixed in 4% paraformaldehyde and then embedded in paraffin after some processing. Two cortex tissue samples (at a volume of $1mm^3$) were immersed in 2.5% glutaraldehyde for later electron microscopic observation. Other cortex tissue samples were stored at –80°C for total RNA extraction.

1.4 Hematoxalin Eosin (HE) Staining

Paraffin-embeded sections (with a thickness of 2 μm) of cortex tissues were dewaxed, stained with hemotoxalin for 3 min, washed with water, differentiated with 1% acid ethanol for a few seconds and then washed with water. Afterwards, the sections were stained with eosin for 30 s, dehydrated, cleared and mounted for light microscopic examination.

1.5 Observation of Ultrastructure of Podocytes by Electron Microscopy

Sampled cortex tissues were fixed first in 2.5% glutaraldehyde for 2 h, and then in osmium tetroxide. Thereafter, they were dehydrated with graded alcohol series, cleared in acetone, 618 resin-embeded, ultrathinly sectioned, stained with uranyl acetate and lead citrate, and observed under electron microscope (TECNAI G2 12, FEI Co., the Netherlands).

1.6 RT-PCR

Total RNA was extracted from kidney cortex tissues using Trizol Reagent according to the manufacturer's instruction, and the RNA concentration was determined after the sample was dissolved in diethylpirocarbonate-treated water. Isolated RNA (1 μg) of each sample was subjected to reverse transcription by using ReverTra Ace (TOYOBO Co., Japan) according to the manufacture's protocol. The resulting cDNA (3 μL) was used for PCR amplification. The sequence-specific primers were designed and synthesized by Shanghai Sangon Biotech Co. Primers used were as follows: heparanase sense:5'-CCCAACAAGGAACCCACCTC-3', antisense: 5'-TGGTCGCAACTCGTCCATTC-3', with product length being 604 bp; β-actin sense: 5'-ATCGTGCG TGACATTAAAGAG-3', antisense: 5'-CCAGGATAGA GCCACCAAT-3' with product length of 418 bp. The PCR amplification was started with 3 min of denaturation at 94 °C, which was followed by 34 cycles (for β-actin, 30 cycles) of denaturation at 94°C for 30 s, annealing at 57°C for 40 s (for β-actin, 53°C for 30 s), and polymerization at 72 °C for 75 s (for β-actin, 30 s). The final extension lasted 7 min at 72°C and then ended at 4°C. PCR products (5 μL) were separated on 1% ethidium bromide-stained agarose gels and later scanned with EC3 Imaging System (UVP, USA).

1.7 Immunhistochemical Staining

Sections, 4-μm thick, were dewaxed, microwaved in 10 mmol/L citrate buffer (pH 6) for 20 min and cooled to room temperature. Then they were washed three times in phosphate-buffered saline (PBS) for 5 min per wash. H_2O_2 (3%) was used to inactivate endogenous hydrogen peroxidase. Sections were washed three times in PBS for 3 min per wash, blocked in 10% goat serum for 30 min and then incubated with primary antibodies (monoclonal rat anti-desmin antibody, from Boster Co., China; polyclonal rabbit anti-CD2AP antibody, from Santa Cruz Co., USA; polyclonal rabbit anti-heparanase antibody, from Boster Co., China) diluted at 1:50 in PBS overnight at 4°C. After routine washes with PBS, biotin-labeled secondary antibody was added at room temperature. After 30-min incubation, HRP-conjugated avidin was given to act for 15 min, which was followed by PBS washes. The sections were afterwards developed with DAB, counterstained with hematoxylin and observed under light microscope. Views of 8 high power fields were randomly selected and absorbance (A) of positive signals was detected by using ImagePro Plus 6.0 software package (Media Cybernetics, USA).

1.8 Statistical Analysis

All data were expressed as $\bar{x}\pm s$. One-way ANOVA analysis with the aid of SPSS l2.0 software package was performed to determine the statistical significance between groups. Correlation of quantitative data was measured by using Pearson correlation analysis with the aid of Excel 2003 software. Statistical significance was defined as $P<0.05$.

2 RESULTS

2.1 Changes in the Excretion of 24-h Urinary Protein

Excretion of 24-h urinary protein at day 14 and 28 after AN induction was increased significantly in the AN group as compared with that in the control group ($P<0.01$ for each). And it was decreased substantially in the sulodexide group. Statistical difference was noted in 24-h urinary protein excretion at day 28 between the AN group and the sulodexide group ($P<0.01$) (fig. 1).

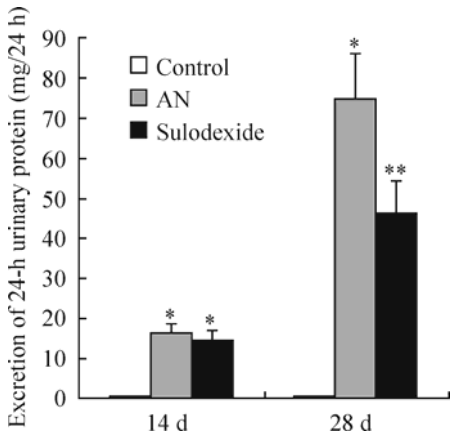

Fig. 1 Excretion of 24-h urinary protein at day 14 and 28 after the model establishment

*$P<0.01$ as compared with the control group; #$P<0.01$ as compared with the AN grouP

2.2 HE Staining Results

No obvious pathological changes were found in the glomeruli of AN rats under light microscopy at day 14 and 28 after AN induction. Numerous protein casts were present in the lumens of renal tubules of AN rats (fig. 2).

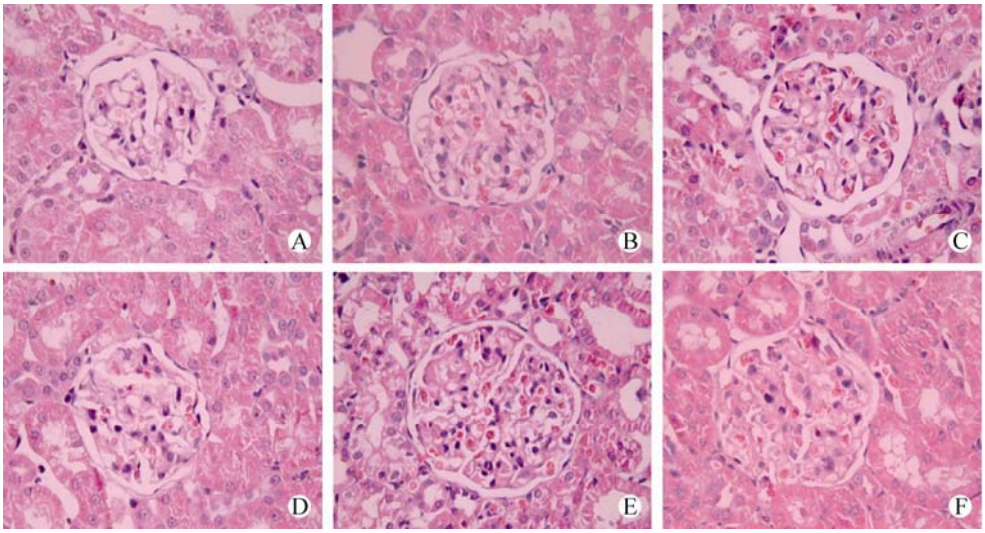

Fig. 2 Pathological changes in the glomeruli by light microscopy (HE staining×400)
A: Control group (14 d); B: AN group (14 d); C: Sulodexide group (14 d);
D: Control group (28 d), E: AN group (28 d); F: Sulodexide group (28 d)

2.3 Morphological Changes in Podocytes under Electron Microscope

Diffuse foot process effacement was found in the podocytes and a large number of lysosomes were present in the cytoplasm of podocytes in the AN group 28 days after the model establishment. Segmental foot process effacement was seen at day 28, and no obvious change was noted in the cytoplasm of podocytes in the sulodexide group (fig. 3).

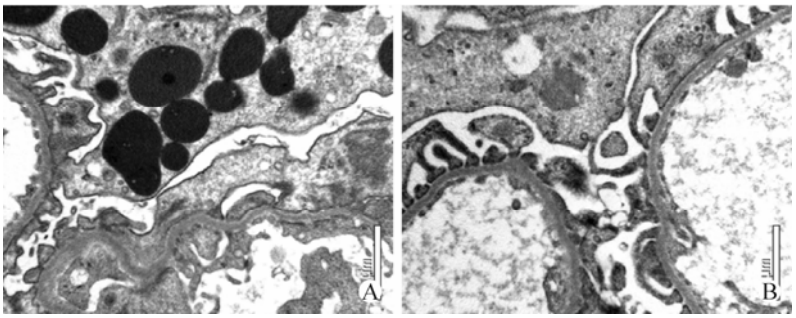

Fig. 3 Pathological changes in podocytes by electron microscopy
A: AN group (28 d); B: Sulodexide group (28 d)

2.4 Expression of Heparanase mRNA

Compared with that in the control group, the expression of heparanase mRNA was significantly increased in kidney cortex tissues in the AN group at day 14 and 28 after AN induction (P<0.01).The expression of heparanase mRNA was decreased substantially in the sulodexide group and significant differences were found in the heparanase mRNA expression between the AN group and the sulodexide group at day 14 and 28 (P <0.01 for each) (fig. 4).

2.5 Expression of Desmin, CD2AP, Heparanase Proteins

Immunhistochemical staining showed that desmin, CD2AP, heparanase mainly expressed in the glomeruli. All the proteins were increased significantly in the AN group (P<0.05) at day 14 and 28 after AN induction as compared with those in the control group. And the expressions of desmin, CD2AP and heparanase in the sulodexide group were significantly lower than those in the AN group at day 14 and 28 (P<0.05 for each) (fig. 4). Correlation analysis showed that the expression of heparanase protein was positively correlated with the expression of desmin, CD2AP protein and 24-h urinary protein excretion (r_1=0.980，r_2=0.984, r_3=0.981, P<0.01) (fig. 5, table1).

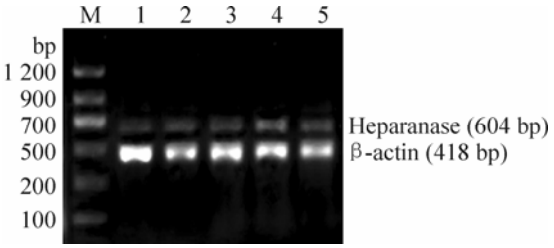

Fig. 4 Heparanase mRNA expression by RT-PCR
M: Marker; 1:Control group (14 d); 2: AN group (14 d), 3: Sulodexide group (14 d); 4: AN group (28 d); 5: Sulodexide group (28 d)

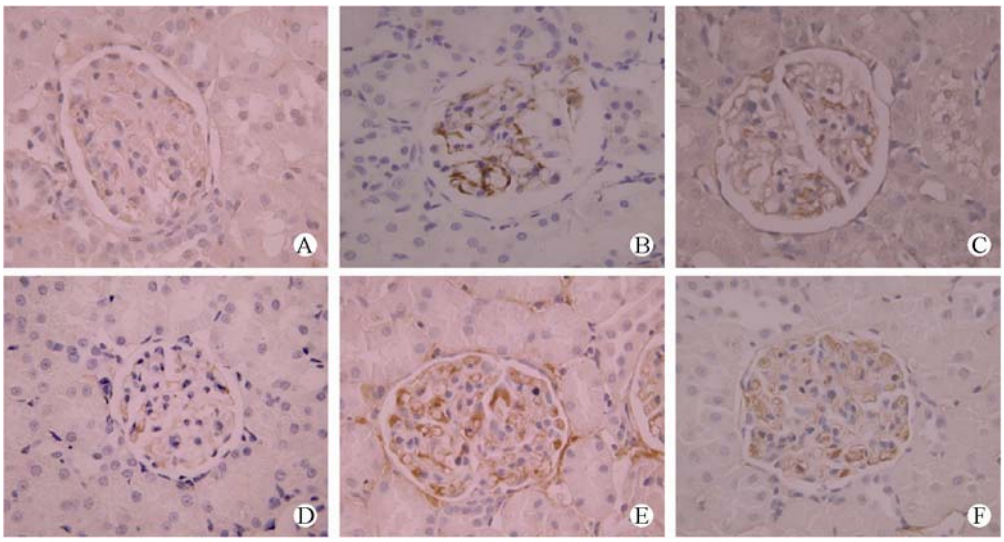

Fig. 5 Desmin protein expression in the cortex tissues (DAB×400)
A: Control group (14 d); B: AN group (14 d); C: Sulodexide group (14 d); D: Control group (28 d); E: AN group (28 d); F: Sulodexide group (28 d)

Table1 Expressions of of desmin, CD2AP, heparanase in the cortex tissues ($\bar{x}$±s, n=6)

Groups	Absorbance (A) values		
	Desmin	CD2AP	Heparanase
Control			
At day 14	40.6±11.9	219.5±14.0	64.2±6.9
At day 28	42.5±9.4	207.3±15.3	67.5±5.0
AN			
At day 14	486.0±21.4*	756.7±25.3*	226.4±24.2**
At day 28	1867.7±195.3*	1885.2±258.1*	446.6±33.1**
Sulodxide			
At day 14	313.2±11.5$^{\#}$	490.6±16.4$^{\#}$	166.0±24.6$^{\#\#}$
At day 28	1194.2±80.8$^{\#}$	1217.5±119.0$^{\#}$	354.6±25.9$^{\#\#}$

$^{*}P$<0.05, $^{**}P$<0.01 vs control; $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs AN

3 DISCUSSION

Our study found that sulodexide could effectively reduce the 24-h urinary protein excretion of AN rats in a time-dependant manner, which suggested that sulodexide may improve the glomerular permeability. It has been demonstrated that GAG-based drugs could maintain the composition of GBM[11], improve the function of vascular endothelia[12], prevent TGF-β1 over-expression in renal tissue[13], and thereby reduce proteinuria. To further elucidate the anti-proteinuric mechanism of sulodexide, we observed the effects of sulodexide on podocyte injury.

Podocyte injury is a hallmark of glomerular diseases[14]. Early podocyte injury shows no obvious morphological changes in podocytes by light microscopy but characteristically presents with podocyte foot process effacement under electron microscope[15]. The results in the present study showed diffuse foot process effacement was found in the AN group by electron microscopy, indicating the success of model establishment. Foot process effacement presented as segmental in the AN rats after the treatment with sulodexide, suggesting that sulodexide helps maintain and restore the morphology and function of podocytes.

Desmin is one of the intermediate filament proteins that expressed in podocytes. It can combine with vimentin and nestin to form a multimer which plays an important role in maintaining podocyte cytoskeleton[16]. Desmin has been found to constantly rise in many kinds of rat models of podocyte injury and is generally believed to be a mark for podocyte injury[17]. Our study showed that the desmin expression in the glomeruli was substantially reduced in the sulodexide-treated rats as compared with that in the AN rats, suggesting sulodexide could alleviate podocyte injury by decreasing the expression of desmin.

It is currently believed that any abnormal changes in the key component of SD complex lead to proteinuria[18]. CD2AP is an important SD component that links other two important SD proteins nephein and podocin[19–20]. Mounting evidence showed that CD2AP played an essential role in the development and progression of proteinuria[21]. Our results showed that CD2AP was increasingly expressed in the glomeruli in the AN rats, suggesting in the early period of podocyte injury, key components of SD are compensatorily increased by the body but still fail to exercise their normal function due to abnormal distribution. In our study, CD2AP expression

was significantly lower in the sulodexide group than in the AN group, which suggested that sulodexide could stabilized CD2AP expression and therefore exert the protective effects on podocyte injury.

Heparanase, a kind of β-D-endoglycosidase, was reported to selectively degrade the negatively charged heparin sulfate (HS) of heparan sulfate proteoglycans(HSPG)[22]. Increased expression of heparanase has been demonstrated in several animal models and human glomerular diseases, such as passive Heymaan nephritis[22], puromycin aminonucleoside-induced nephrosis[23], diabetic nephropathy[24] and minimal change disease[25]. And heparanase expression was mainly confined to podocytes in glomerulus. GAG -based drugs were found to inhibit the enzymatic activity of heparanase[3]. The results in the present study showed the expression of heparanase mRNA and protein was low in the control group but increased significantly in the AN group. After the treatment with sulodexide, the expression of heparanase in the AN rats was decreased. These results indicated that sulodexide could alleviate podocyte injury by inhibiting heparanase expression.

Prevention and treatment of podocyte injury and amelioration of proteinuria are important intervention strategies for early glomerular diseases. Our study showed sulodexide could alleviate podocyte injury. The finding provides theoretical evidence for the use of sulodexide in the treatment of glomerular diseases, yet the molecular mechanism of sulodexide awaits further investigation.

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