

Low molecular weight heparin may benefit nephrotic remission in steroid-sensitive nephrotic syndrome via inhibiting elastase

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Abstract. Low molecular weight heparin (LMWH) has a structure similar to heparan sulfate, which exerts anti-inflammatory effects via inhibiting elastase (Ela) activity. Release of Ela along the glomerular capillary wall may induce glomerular injury and proteinuria. The present study aimed to investigate the influence of LMWH on steroid-sensitive nephrotic syndrome (SSNS) and the potential underlying mechanism. A total of 40 SSNS patients and 20 healthy controls were recruited. SSNS patients were treated with LMWH and prednisone simultaneously (LMWH+pred group) or with prednisone alone (pred group). Proteinuria, urinary glycosaminoglycans (GAGs), serum Ela and urinary creatinine levels were measured. The nephrotic period of SSNS was 15.93±5.78 days. The nephrotic period of SSNS in LMWH+pred group was significantly reduced compared with the pred group (14.13±4.56 vs. 18.63±6.49 days; $P<0.05$). At the follow-up of the SSNS patients, there was no statistically significant difference in number of relapses between the LMWH+pred and pred groups. Proteinuria (2.51±0.97 g/24 h), urinary GAG levels (4.92±0.87 mg/mmol creatinine) and serum Ela levels (77.64±10.99 ng/l) were significantly greater in the nephrotic period of SSNS compared with the remission period (0.107±0.026 g/24 h, 1.53±0.27 mg/mmol Cr and 41.92±7.81 ng/l, respectively) and the healthy control group (0.098±0.027 g/24 h, 1.40±0.26 mg/mmol creatinine and 38.43±9.83 ng/l, respectively; $P<0.05$). During the remission

period, urinary GAG and serum Ela levels in the LMWH+pred group were significantly reduced compared with the pred group ($P<0.05$), whereas proteinuria did not differ between these groups ($P>0.05$). Positive correlations were revealed between urinary GAG excretion and proteinuria ($r=0.877$; $P<0.05$), proteinuria and serum Ela levels ($r=0.844$; $P<0.05$) and serum Ela levels and urinary GAG excretion ($r=0.881$; $P<0.05$). The results of the present study indicated that elevated serum Ela levels may induce proteinuria by degrading GAGs in the glomerular basement membrane in children with SSNS. LMWH may benefit nephrotic remission of SSNS via inhibiting Ela.

Introduction

Primary nephrotic syndrome (PNS) is a common renal disease in children. The majority of cases are steroid-sensitive (SSNS), the prognosis of which is good; however, patients are prone to relapse (1). The pathogenesis of PNS remains unclear; however, dysfunction of the charge-selectivity of the glomerular basement membrane (GBM) may contribute to proteinuria (2). The anionic filtration of the GBM involves glycosaminoglycans (GAGs), particularly heparan sulfate proteoglycan (HSPG), a highly anionic molecule that regulates the charge- and size-selective aspects of glomerular permselectivity (3). GAGs are linear polymers of repeating amino sugar uronic acid disaccharides produced by and associated with the majority of mammalian cells and certain bacterial cells (4). GAGs may interact with numerous proteins and participate in matrix organization, cell adhesion, differentiation, growth, apoptosis and mouse kidney development (5,6). Certain studies have demonstrated that reduced GAGs content in the GBM results in increased passage of proteins across the GBM, particularly negatively charged proteins (7,8). Urinary GAGs levels have been suggested as a marker of glomerular damage in PNS (9,10). GAGs in the GBM may be degraded by certain proteinases, including heparanase or elastase (Ela) (11). Klebanoff *et al* (12) revealed that Ela may degrade subendothelial matrix HSPG *in vitro*, which may be involved in proteinuria. By contrast, studies have demonstrated that proteinuria may be alleviated via the administration of GAGs (13-15). Low molecular weight heparin (LMWH) is a negatively charged glycoprotein due to

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N-sulfate and O-sulfate on the carbon chain and has a similar structure to HSPG. Previous studies have revealed that the anti-inflammatory effects of LMWH were not dependent on its anticoagulant activities, but on inhibiting Ela activity and adhesion of neutrophils to vascular endothelial cells, which may have therapeutic potential for the treatment of chronic inflammatory diseases (16-18).

The influence of LMWH on urinary GAG and serum Ela levels in children with SSNS remains to be determined. Therefore, the present study used LMWH as a convenient analogue of HSPG for experimental purposes to investigate the influence of HSPG on urinary protein excretion and serum Ela levels. The results of the present study may facilitate the elucidation of the association between urinary GAGs, proteinuria and Ela.

Patients and methods

Patients and methods. Children hospitalized as a result of SSNS and healthy controls from the outpatient department, were recruited for the present study from the West China Second University Hospital (Chengdu, China) between March 2010 and July 2014. A total of 40 SSNS patients (male, n=26; female, n=14; age, 7.19 ± 3.02 years) and 20 sex and age-matched healthy controls (male, n=11; female, n=9; age, 7.32 ± 3.48 years) were included in the present study. All of the SSNS patients had PNS, as diagnosed according to the criteria from the International Study of Kidney Disease in Children (19). Urine protein excretion >50 mg/kg/24 h was regarded as the nephrotic state, whereas <4 mg/kg/24 h was regarded as the remission phase. PNS patients with complications were excluded from the present study. The coagulation function in all SSNS patients was normal. It was defined as achieving remission within 4 weeks of treatment with 2 mg/kg/day (with the majority of doses being 60 mg/day) prednisone (pred). SSNS patients were divided into two groups, which were similar in sex and age. The LMWH+pred group (n=24) was treated with pred and LMWH (30-50 IU/kg/day) simultaneously, and the pred group (n=16) was treated with pred only. The two groups received similar assistant therapy, including captopril and calcium. Follow-up began on the date of diagnosis and ended in July 2014. The median follow-up was 325 days (range, 209-451 days). Regular history, physical and laboratory examinations were performed on all patients every one to two months following the disappearance of proteinuria. The present study was approved by the Institutional Review Board of West China Second University Hospital, Sichuan University (Sichuan, China), and blood and urine samples of SSNS patients and healthy controls were obtained and handled in accordance with its guidelines. The present study was performed in accordance with the ethical standards of the Declaration of Helsinki. All parents or guardians signed informed consent.

Measurement of proteinuria excretion, urinary GAG excretion and serum parameters. In SSNS patients, two urine samples were collected during the nephrotic and remission phases. In the healthy controls, one urine sample was obtained from each individual; 24 h urine samples were collected and stored at -80°C until analysis. Urinary proteins were measured by the pyrogallol end-point method (10), while urinary GAGs

excretion was examined using the modified Whiteman process (10,11). GAGs levels were calculated using a calibration curve with HS as a standard, and corrected with urinary creatinine (Cr).

At the same time, blood samples (4 ml) were collected from SSNS patients and healthy controls and stored in tubes containing EDTA to prevent coagulation, and subsequently centrifuged at $400 \times g$ for 5 min at 4°C . Enzymatic activity of Ela was quantified by ELISA according to the manufacturer's protocols (ab119553; Abcam, London, Cambridge, UK).

Urine levels of Creatinine (Cr) were measured using an Hitachi 7600 Chemistry Analyzer (Hitachi, Ltd., Tokyo, Japan).

Statistical analysis. All statistical analyses were performed in SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Continuous data were presented as the mean \pm standard deviation. Comparison of continuous data was performed with an independent t-test between the two groups, whereas the correlation among categorical variables was analyzed by a two-sided Chi-square test. Multiple groups were compared using one way Analysis of variance. Spearman's test was used to analyze correlations. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient clinical profile. A total of 40 children hospitalized with SSNS and 20 healthy controls were enrolled in the present study. The clinical characteristics of the participants are presented in Table I. Of the 40 SSNS patients, 25 patients had an infection history 7 to 14 days prior to the diagnosis of nephrotic syndrome (upper respiratory tract infection, n=6; acute tonsillitis, n=10; bronchitis, n=5; pneumonia, n=4; Fig. 1A). In the pred group, 10 patients had an infection (upper respiratory tract infection, n=2; acute tonsillitis, n=5; bronchitis, n=2; pneumonia, n=1). In the LMWH+pred group, 15 patients had an infection (upper respiratory tract infection, n=3; acute tonsillitis, n=7; bronchitis, n=3; pneumonia, n=2).

The nephrotic period of SSNS was 15.93 ± 5.78 days (range, 6-27 days). The nephrotic period of SSNS in the LMWH+pred group was 14.13 ± 4.56 days, which was significantly reduced compared with the pred group (18.63 ± 6.49 days, Table I).

All patients were followed up for an average of 325 days (range from 209 to 451 days). During the follow-up period, there were 31 relapse cases of SSNS. Of these cases, 3 were of frequent relapse; one in the LMWH+pred group, whose renal pathology indicated minimal change disease, and two in the pred group, whose renal pathology indicated minimal change disease and focal segmental glomerulosclerosis. As presented in Fig. 1B, the 31 relapse cases of SSNS were suspected to be the result of infection (n=21, 67.7%), reducing pred dose (n=7, 22.6%) or no clear reason (n=3, 9.7%). In the 18 relapse patients in the LMWH+pred group, the suspected factors were infection (n=12, 66.7%), reducing pred dose (n=4, 22.2%) or no clear reason (n=2, 11.1%). In the 13 relapse patients in the pred group, the suspected factors were infection (n=9, 69.2%), reducing pred dose (n=3, 23.1%) or no clear reason (n=1, 7.7%). There was no statistically significant difference in number of relapses between the LMWH+pred and pred groups ($P > 0.05$, Table I).

Table I. Clinical profiles of 40 SSNS patients and 20 healthy controls.

Parameter	SSNS patients (n=40)			Healthy controls (n=20)		
	Pred (n=16)	LMWH+pred (n=24)	P-value	All SSNS (n=40)	All control (n=20)	P-value
Age (years)	7.43±3.52	7.02±2.69	0.092 ^a	7.19±3.02	7.32±3.48	0.214 ^a
Gender			0.685 ^b			0.453 ^b
Male	11 (68.8%)	15 (62.5%)		26 (65.0%)	11 (55%)	
Female	5 (31.2%)	9 (37.5%)		14 (35.0%)	9 (45%)	
Respiratory infection			1 ^b			/
No	6 (37.5%)	9 (37.5%)		15 (37.5%)	0	
Yes	10 (62.5%)	15 (62.5%)		25 (62.5%)	0	
LMWH			/			/
No	16 (100%)	0 (0%)		16 (40%)	0	
Yes	0 (0%)	24 (100%)		24 (60%)	0	
Relapse			0.643 ^b			/
No	3 (18.8%)	6 (25.0%)		9 (22.5%)	0	
Yes	13 (81.2%)	18 (75.0%)		31 (77.5%)	0	
Nephrotic period (days)	18.63±6.49	14.13±4.56	0.014 ^a	15.93±5.78	0	

Data for age and nephrotic period are presented as the mean ± standard deviation. ^aCalculated with an independent t-test; ^bcalculated with a two-sided Chi-square test. Pred, prednisone; LMWH, low molecular weight heparin; SSNS, steroid-sensitive nephrotic syndrome.

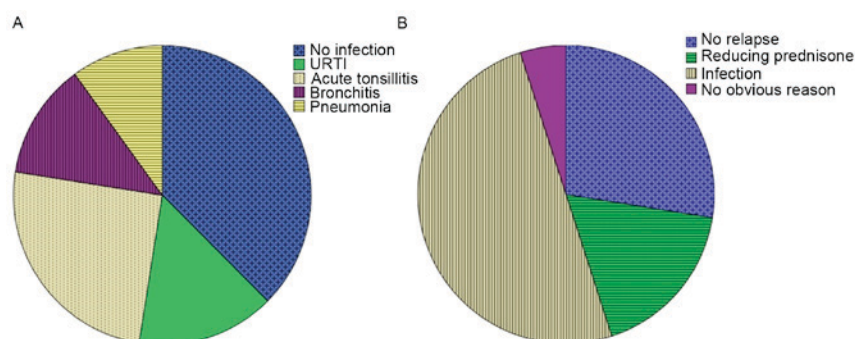


Figure 1. (A) Distribution of potential inducing factors in SSNS patients and (B) the reasons for relapse. URTI, upper respiratory tract infection.

Proteinuria in 24 h. As presented in Fig. 2, proteinuria in the nephrotic period of SSNS (2.51 ± 0.97 g/24 h) was significantly greater compared with the remission period of SSNS (0.107 ± 0.026 g/24 h) and the healthy control group (0.098 ± 0.027 g/24 h, $P < 0.05$), whereas proteinuria was not significantly different between the remission period of SSNS and the healthy control group ($P > 0.05$, Fig. 2A). In the nephrotic period of SSNS, proteinuria in the LMWH+pred group was similar to the pred group (2.62 ± 0.99 g/24 h vs. 2.34 ± 0.94 g/24 h, $P > 0.05$, Fig. 2B). In the remission period of SSNS, there were no significant differences in proteinuria between the LMWH+pred (0.102 ± 0.024 g/24 h), pred (0.114 ± 0.029 g/24 h) and healthy control groups (0.098 ± 0.027 g/24 h, $P > 0.05$, Fig. 2C).

Urinary GAG excretion in 24 h. The results of urinary GAG excretion were corrected by the ratio of urinary GAGs (UGAGs) to urinary Cr (UCr). As presented in Fig. 3, in SSNS

patients the ratio of UGAGs/UCr in the nephrotic period (4.92 ± 0.76 mg/mmol Cr) was significantly greater compared with the remission period (1.39 ± 0.14 mg/mmol Cr) and the healthy control group (1.37 ± 0.10 mg/mmol Cr; $P < 0.05$), whereas the ratio of UGAGs/UCr in the remission period was similar to the control group ($P > 0.05$; Fig. 3A). Comparison of the LMWH+pred and pred groups revealed no significant difference in the ratio of UGAGs/UCr in the nephrotic period (4.95 ± 0.71 vs. 4.89 ± 0.85 mg/mmol Cr; $P > 0.05$; Fig. 3B). In the remission period, the ratio of UGAGs/UCr in the LMWH+pred group was significantly reduced compared with the pred group (1.36 ± 0.12 vs. 1.45 ± 0.15 mg/mmol Cr; $P < 0.05$), whereas these two groups were not significantly different compared with the healthy control group ($P > 0.05$; Fig. 3C). Therefore, following steroid therapy in SSNS patients, there was a significant reduction in GAG excretion, and pred accompanied by LMWH treatment was more efficient in reducing urinary GAGs excretion compared with pred monotherapy.

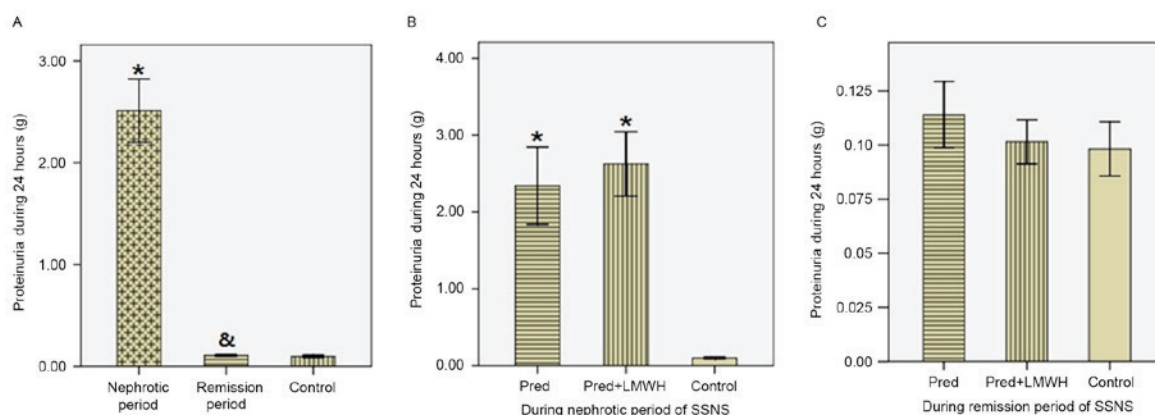


Figure 2. Proteinuria in 24 h. (A) Effect of treatment in SSNS patients compared with healthy controls. Proteinuria during the (B) nephrotic and (C) remission periods of SSNS. * $P < 0.05$ vs. control; # $P < 0.05$ vs. nephrotic period. Pred, prednisone; LMWH, low molecular weight heparin; SSNS, steroid-sensitive nephrotic syndrome.

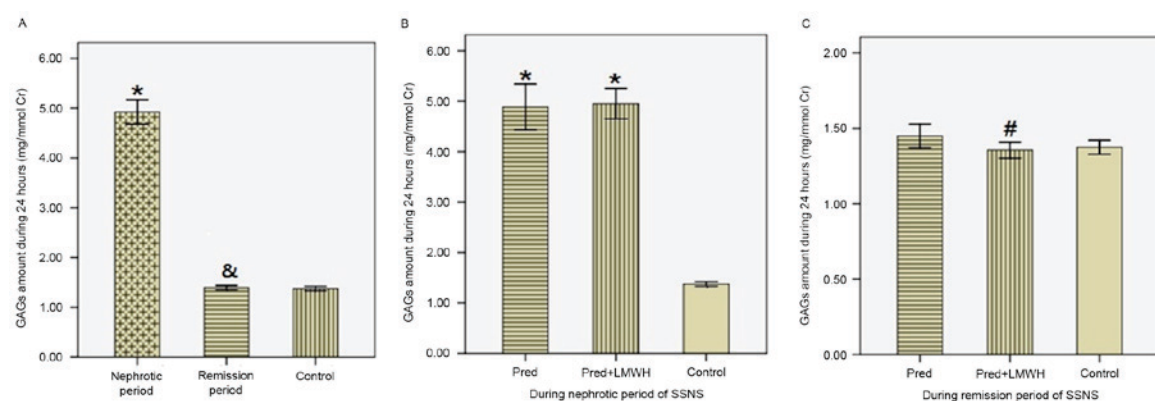


Figure 3. Urinary GAG excretion in 24 h. (A) Effect of treatment in SSNS patients compared with healthy controls. Urinary GAG excretion during the (B) nephrotic and (C) remission periods of SSNS. * $P < 0.05$ vs. control; # $P < 0.05$ vs. nephrotic period; # $P < 0.05$ vs. pred. Pred, prednisone; LMWH, low molecular weight heparin; SSNS, steroid-sensitive nephrotic syndrome; GAG, glycosaminoglycan; Cr, creatinine.

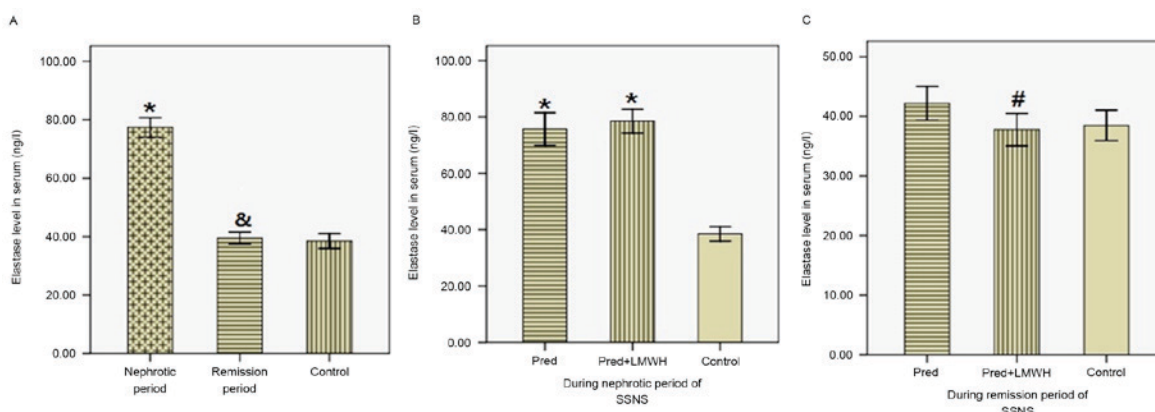


Figure 4. Serum elastase levels. (A) Effect of treatment in SSNS patients compared with healthy controls. Serum levels of elastase during the (B) nephrotic and (C) remission periods of SSNS. * $P < 0.05$ vs. control; # $P < 0.05$ vs. nephrotic period; # $P < 0.05$ vs. pred. Pred, prednisone; LMWH, low molecular weight heparin; SSNS, steroid-sensitive nephrotic syndrome.

Serum Ela levels. As presented in Fig. 4, serum Ela levels in the nephrotic period (77.39 ± 10.38 ng/l) were significantly greater compared with the remission period (39.55 ± 6.35 ng/l, $P < 0.05$) and the healthy control group (38.48 ± 5.44 ng/l, $P < 0.05$), whereas Ela levels in the remission period were similar to those in the control group (Fig. 4A). Ela levels were not significantly

different between the LMWH+pred and pred group during the nephrotic period (78.53 ± 10.05 vs. 75.69 ± 10.96 ng/l, $P > 0.05$, Fig. 4B). In the remission period, Ela levels in the LMWH+pred group were significantly reduced compared with the pred group (37.78 ± 6.49 vs. 42.20 ± 5.28 ng/l, $P < 0.05$), whereas these two groups were not significantly different compared with the

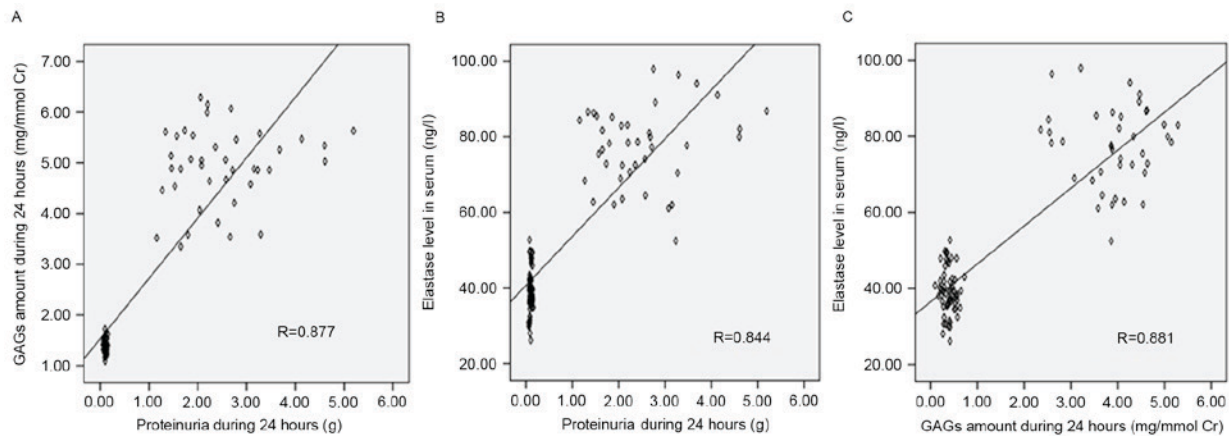


Figure 5. Correlations between urinary GAG excretion, proteinuria and serum elastase levels. Correlation between (A) urinary GAG levels and proteinuria, (B) serum elastase levels and proteinuria and (C) serum elastase levels and urinary GAG excretion. GAG, glycosaminoglycan; Cr, creatinine.

healthy control group ($P>0.05$, Fig. 4C). Therefore, following steroid therapy in SSNS patients, there was a significant reduction in serum Ela levels, and pred accompanied by LMWH treatment was more efficient in reducing serum Ela levels compared with pred monotherapy.

Association between urinary GAG excretion, proteinuria and serum Ela levels. Correlation analysis of all participants revealed significant positive correlations between urinary GAG levels and proteinuria ($r=0.877$, $P<0.05$, Fig. 5A), serum Ela levels and proteinuria ($r=0.844$, $P<0.05$, Fig. 5B), and serum Ela and urinary GAG levels ($r=0.881$, $P<0.05$, Fig. 5C).

Discussion

The results of the present study suggested that pred and LMWH treatment reduced proteinuria and urinary GAGs levels and decreased the nephrotic period in SSNS patients, but had no effect on SSNS relapse.

It had previously been reported that the GAGs content of GBM was reduced in nephrotic syndrome and that this decrease in GAGs induced the loss of the GBM negative charge, which might contribute to an increase in urinary proteins (20,21). Girardin *et al* suggested that various lymphocytic factors may decrease the anionic charge of the GBM, and so increase permeability to albumin (22). Mitsuhashi *et al* (23) investigated adult patients with minimal change disease and revealed that they excreted significantly greater levels of HSPG compared with controls. Consistent with these results, in child SSNS patients, the present study detected elevated levels of urinary GAGs excretion during the nephrotic period and normal levels during the remission period. Heeringa *et al* (24) reported that in rats, renal perfusion with Ela might induce proteinuria by GBM HSPG degradation. Our previous studies of rats infected with respiratory syncytial virus (RSV) indicated that RSV might induce renal minimal change similar to human minimal change disease, and degradation of GAGs on GBM potentially via elevated heparanase and Ela (25-27). The present study demonstrated that LMWH administration may reduce excretion of urinary proteins and that pred accompanied by LMWH treatment was

more efficient compared with pred monotherapy in reducing nephrotic period and urinary GAGs excretion, and decreasing the level of serum Ela. Previous studies have reported that LMWH may inhibit the release of Ela from human neutrophils (28,29). Therefore, LMWH may be beneficial in SSNS via inhibiting Ela and reducing degradation of GBM GAGs. The present study also revealed that LMWH had no preventive and predictive effect on the relapse of SSNS.

In conclusion, the results of the present study indicated that urinary excretion of GAGs was associated with the degree of proteinuria and serum Ela levels in children with SSNS. Treatment with LMWH may benefit proteinuria remission.

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