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Effects of sequential application of immunosuppressive agents according to the cell cycle in adriamycin-induced nephropathy rats

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Abstract. – OBJECTIVE: The aim of this study was to observe the therapeutic effects of three sequential drug-based treatments according to the cell cycle in rats with adriamycin-induced nephropathy.

MATERIALS AND METHODS: A rat model of adriamycin-induced nephropathy was prepared by two injections, and three experimental groups were set up: control group (n=8); adriamycin-induced nephropathy rat group (n=8); and Meprednisone (MP), Ciclosporin (CsA), and mycophenolate (MMF) treatment group (n=8). Twenty-four-hour urine protein was quantified and serum total protein (TP), albumin (ALB), cholesterol (Chol), triglyceride (TG), urea nitrogen (BUN), and serum creatinine (Scr) were measured by an automatic biochemical analyzer. Pathological changes in renal tissues were observed by light microscopy. Serum matrix metalloproteinase-2 (MMP-2), MMP-9, and transforming growth factor-β1 (TGF-β1) were evaluated by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). Connective tissue growth factor (CTGF) expression was measured by Western blotting. Expression of nephrin and podocin in podocytes was compared by immunohistochemistry and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RESULTS: Compared with the control group, 24-h urine protein in nephropathy group was significantly reduced at 2, 4, 8, and 12 weeks ($p<0.01$). Twenty-four-hour urine protein in the three treatment groups was significantly decreased compared with nephropathy group at 8 and 12 weeks ($p<0.05$). There were no significant differences among treatment groups ($p>0.05$), but their levels were higher than those in control group. TP and ALB levels in nephropathy group were decreased compared with control group ($p<0.01$) and increased compared with treatment groups ($p<0.05$), while TG and Chol levels in nephropathy group were increased compared with control group ($p<0.01$) and decreased compared with treatment group ($p<0.05$). There were no significant differences in biochemical parameters among the treatment groups. TGF-β1 levels were decreased, MMP-2 and MMP-9 levels were increased, and CTGF expression was reduced in the three therapeutic groups. Among the treatment groups, the combination of MP, CsA, and Rapa significantly inhibited fibrosis. The protein and mRNA levels of nephrin and podocin were significantly decreased in nephropathy group and their expression and distribution were partially restored in treatment groups.

CONCLUSIONS: The present findings suggest that the sequential therapeutic treatments based on the cell cycle significantly improved the pathological changes in adriamycin-induced nephropathy rats. The sequential treatments significantly reduced urine protein levels, increased TP, ALB, MMP-2, and MMP-9 levels, decreased TG, Chol, and TGF-β1 levels, restored expression of nephrin and podocin in renal tissues, and significantly improved renal fibrosis.

Key Words:
Sequential application, Immunosuppressive agents, Cell cycle, Adriamycin-induced nephropathy.

Introduction

In recent years, application of immunosuppressive agents to the treatment of glomerular diseases has markedly developed. New drugs are emerging, and combinations of drugs are preferred to avoid long-term adverse effects and serious consequences of hormone therapy and immunosuppressive agents[1]. Therefore, treatment plans involving combined use of immunosuppressive agents have also diversified. Treatment according to the cell cycle is based on the principle of cell dynamics, with use of sequential combinations of cell cycle-specific drugs and/or cell cycle non-specific drugs, and selection of drugs according to different cell cycles to improve the therapeutic effect. Intermittent administration according to the cell cycle reduces

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the occurrence of adverse reactions[2]. In this study, three drug regimens were chosen to treat adriamycin-induced nephropathy in rats. The efficacies of the regimens were observed and their mechanisms of action were explored. The study provides an experimental basis for sequential clinical treatments of glomerular diseases according to the cell cycle.

Materials and Methods

Materials

Male Sprague-Dawley rats weighing 180-220 g were purchased from the Experimental Animal Center of Shanxi Medical University. The animal certificate number was SCXK (Jin) 2017-0001. The rats were kept at constant temperature (22°C±1°C) and relative humidity (65%-70%) on a 12-h/12-h light/dark cycle. The experiments were performed after 1 week of feeding. This investigation was approved by the Animal Ethics Committee of Shanxi Medical University Animal Center.

Adriamycin was purchased from Zhejiang Hairong Pharmaceutical (Hangzhou, China). Meprednisone (MP) was purchased from Pharmacia Italia SpA. Ciclosporin (CsA), mycophenolate (MMF), tacrolimus (FK506), and rapamycin (Rapa) were obtained from North China Pharmaceutical (Shijiazhuang, China). Cyclophosphamide (CTX) for injection was purchased from Shanxi Pude Pharmaceutical Co. Ltd. (Datong, China).

Sulfosalicylic acid was purchased from the Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Rat matrix metalloproteinase-2 (MMP-2) enzyme-linked immunosorbent assay (ELISA) Kit, Rat MMP-9 ELISA Kit, Rat transforming growth factor-β1 (TGF-β1) ELISA Kit, rabbit anti-nephrin antibody, rabbit anti-podocin antibody, and rabbit anti-CTGF antibody were purchased from Wuhan Bude Biological Engineering Co. Ltd. (Wuhan, China). Two-step immunohistochemical detection reagent, Western blot Test Kit, and diaminobenzidine (DAB) Kit were purchased from Beijing Zhongshan Jinqiao Co. Ltd. (Beijing, China). SV Total RNA Isolation System was purchased from Promega (Madison, WI, USA). SYBR® Prime Script™ RT-PCR Kit (Perfect-RealTime) and DNA markers were purchased from TaKaRa (Otsu, Shiga, Japan).

Preparation and Grouping of Animal Models

Forty male Sprague-Dawley rats were randomly divided into five groups: normal control group (n=8), nephropathy group (n=8), hormone+CsA+MMF treatment group (n=8), hormone+FK506+CTX treatment group (n=8), and hormone+CsA+Rapa treatment group (n=8). The urine protein characteristics of the rats were determined by the sulfanilic acid method before the experiments and all results were negative. Drugs were administered to rats twice by tail vein injection, with first dose at 6 mg/kg and second dose at 4 mg/kg after 1 week. Saline was injected in the control group. For the hormone+CsA+MMF treatment group, CsA (5 mg/kg/d) was administered into the stomach for 1 week after the fourth week of the experiment, followed by MMF (20 mg/kg/d) for 1 week. The 2-week treatment was set as one treatment cycle, and the rats were euthanized after four treatment cycles (end of week 12). For the hormone+FK506+CTX treatment group, FK506 (1 mg/kg/d) was administered into the stomach for 1 week after the fourth week of the experiment, followed by intraperitoneal injection of CTX (20 mg/kg/d) once every other day for 1 week. The 2-week treatment was set as one treatment cycle, and the rats were euthanized after four treatment cycles (end of week 12). For the hormone+CsA+Rapa treatment group, CsA (5 mg/kg/d) was administered into the stomach for 1 week after the fourth week of the experiment, followed by Rapa (2 mg/kg/d) for 1 week. The 2-week treatment was set as one treatment cycle, and the rats were euthanized after four treatment cycles (end of week 12). MP (0.6 mg/kg) was administered into the stomach of all three treatment groups every day, and the same amount of distilled water was administered to the stomach of all other groups until the end of the experiment as a control.

Specimen Collection

Rats were placed in separate metabolic cages on the day before the start of the experiment, and 24-h urine samples were collected. The rats in nephropathy group and treatment groups were also placed in separate metabolic cages at the end of weeks 2, 4, 8, and 12. Their 24-h urine samples were collected and urine protein was quantified by centrifugation. After the rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g), blood samples (5 mL) were collected from the abdominal aorta and centrifuged at 3000 rpm for 10 min to collect serum. The serum was extracted and stored at −80°C. The kidneys were removed immediately after irrigation with saline and the cortex was obtained. Part of the cortex was fixed in formalin solution, and the remainder was stored at −80°C for later analysis. The rats in the control group were also placed in separate metabolic cages at the end of weeks 2, 4, 8, and 12, and euthanized by the module method.

Detection of Parameters

Urine protein quantification, and blood biochemical parameters of serum total protein (TP), albumin (ALB), cholesterol (Chol), triglyceride (TG), urea nitrogen (BUN), and serum creatinine (Scr) were detected by an LX20 automatic biochemical analyzer. The kidney specimens were fixed with 4% paraformaldehyde at 4°C for 24 h, gradually dehydrated by immersion in 75%-100% ethanol, placed in wax, embedded in paraffin, sectioned at 3-μm thickness, stained with periodic acid-Schiff (PAS), and observed by light microscopy.

Detection of Serum MMP-2, MMP-9, and TGF-β1 Levels by Double-Antibody Sandwich ELISA

(1) The kit and samples were allowed to equilibrate at room temperature before the analysis. (2) Eight standard wells were set up, and 50 μL of dilution solution was added to each well. Fifty microliters of standard were added to the first well and mixed, before 50 μL was removed from the well and added to the second well. The dilution was repeated until the seventh well, and finally 50 μL was removed from the seventh well and discarded. The eighth well was a blank control. Next, 50 μL of sample was added to each well, giving a total volume of 100 μL/well. (3) Twenty-five microliters of enzyme-linked substrate was added into the standard and test wells, and mixed. (4) After incubation at 37°C for 60 min, the liquid in each well was discarded, and the wells were washed five times. (5) Fifty microliters of coloring solution A and 50 μL of coloring solution B were separately added to each well and mixed. The reaction was allowed to proceed in the dark for 15 min at room temperature. (6) The blue reaction solution showed a distinct gradient. Fifty microliters of stop solution were added into each well, and mixed. The solution turned yellow and the reaction was stopped. (7) Optical density (OD) values were determined with a microplate reader at 450 nm. (8) A standard curve was created with the standard concentrations on the X-axis and semi-logged OD values on the Y-axis. (8) The corresponding contents of MMP-2, MMP-9, and TGF-β1 were measured by reference to the standard curve based on the OD values of the samples.

Measurement of CTGF Expression in Renal Tissues by Western Blotting

Half a millimeter of freshly-made protein lysis buffer (50 mM Tris-HCl pH 6.8, 15 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF) was added to centrifuged cell pellets, and the samples were passed through a 1-mL syringe 100 times to disrupt and release DNA. After addition of an equal volume of 2× SDS buffer (100 mM Tris-HCl, 200 mM DTT, 20% glycerol, 0.2% bromophenol blue, 4% SDS), the samples were boiled for 5 min, immediately cooled in an ice bath, and centrifuged at 10,000×*g* for 10 min at 10°C. The supernatants were collected. Next, 40 μg of tissue homogenate protein was separated by 8% and 10% gel electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk at 4°C for 1 h, washed, and sequentially incubated with rabbit anti-rat CTGF primary antibody (1:400) and ALP-labeled goat anti-rabbit IgG secondary antibody (1:1000). Finally, ALP developer was used for membrane development. The absorbance of immune complexes on the membranes was measured by an electrophoresis gel imaging analysis system.

Detection of Nephrin and Podocin Expressions by Immunohistochemistry

Paraffin sections of renal tissues were dewaxed, hydrated, and rinsed in phosphate-buffered saline (PBS) for 15 min at room temperature. Rabbit anti-nephrin and mouse anti-podocin primary antibodies (1:200) were added to the samples and incubated for 2 h at 37°C after high-pressure repair. An enhancer was added and incubated for 20 min. The sections were then incubated with appropriate secondary antibodies for 30 min at 37°C. DAB was used for color development, and the samples were observed after mild hematoxylin counterstaining, dehydration, transparency, and sealing. The appearance of brownish-yellow granules in the cytoplasm was considered a positive result. PBS instead of a primary antibody was used as a negative control. An HPIAS-1000 image analysis system was used to perform semi-quantitative analysis of the glomerular immunohistochemistry results. Each specimen was examined in 10 high-magnification fields, and integrated OD values indicated the relative contents of positive staining.

Detection of Nephrin and Podocin mRNA Expressions in Renal Tissues by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Extraction of Total RNA from Kidney Tissues

Total RNA was extracted from rat kidney tissues using TRIzol reagent in accordance with the instruction manual. The rat tissues were ground

in liquid nitrogen, followed by addition of 1 mL of TransZol per 100 mg of tissue. Each sample was homogenized with a homogenizer, repeatedly passed through a pipet, and incubated at room temperature for 5 min. Next, 0.2 mL of chloroform was added per 1 mL of TransZol, shaken vigorously for 15 s, incubated at room temperature for 3 min, and centrifuged at 10,000×g for 5 min at 4°C. The supernatant was transferred to a new microcentrifuge tube. After addition of 500 µL of isopropanol per 1 mL of TransZol and incubation at room temperature for 10 min, the supernatant was discarded. A gelatinous precipitate remained on the side and bottom of the tube. One milliliter of 75% ethanol in DEPC-treated water was added, vigorously vortexed, and centrifuged. After the supernatant was discarded, the precipitate was dissolved in 100 µL of RNA solution, incubated at 55°C-60°C for 10 min, and stored at -80°C until analysis.

Quantification and Purity Detection of Total RNA

After dissolving 5 µL of RNA solution in 500 µL, the OD values of the samples were measured in an ultraviolet spectrophotometer at 260 nm and 280 nm. The RNA concentration was calculated according to the following equation: RNA concentration (µg/mL) = OD260/OD280×40 (µg/mL) × dilution factor. RNA purity was evaluated by the ratio of OD260/OD280. The normal range was 1.8ò-2.0. The extracted RNA was of high purity with little protein contamination.

Primer Design

The primers were designed in GenBank, verified by Primer software, and synthesized by TaKaRa Company (Otsu, Shiga, Japan) The sequences are shown below (Table I).

Reverse Transcription

The RT reaction solution comprised: 1× Primescript™ Buffer (RealTime), 2 µL; PrimeScript™ RT Enzyme Mix l, 0.5 µL; oligo dT primer (50 µM), 0.5 µL; random 6mers (100 µM), 0.5 µL; total RNA, 300 ng; RNase-free dH_2O, 10 µL. cDNA was obtained by one cycle of 70°C for 5 min, 37°C for 60 min, and 70.5°C for 15 min. The components other than the template were gently mixed to avoid bubbles. Each experiment had a blank control (no cDNA temple) and was performed in duplicate. The reaction system was supplemented with RNase-free dH_2O. The real-time PCR was performed in an Mx300P fluorescence quantitative analyzer (Stratagene, La Jolla, CA, USA) using the following conditions: Stage 1: 95°C for 10 s; Stage 2: 40 cycles of 95°C for 5 s and 60°C for 31 s; Stage 3: dissociation. After the reaction, a computer generated the results automatically. The results were calculated by the following formula: test/control=$2^{-\Delta\Delta Ct}$. In the formula, Ct was the number of cycles required for the fluorescence to reach the fluorescence threshold, and ΔΔCt=(Ct target gene–Ct internal reference gene) test–(Ct target gene–Ct internal reference gene) control, to obtain the value of the target gene relative to the internal reference gene. Because the results were calculated exponentially, statistical analysis of ΔCt=Ct target gene–Ct internal reference gene was performed.

Statistical Analysis

Data were recorded in Excel software (Microsoft, Redmond, VA, USA). Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test

Table I. Primer sequences.

Gene	Primer sequence (5'Ð3)	Length (bp)
NPHS1	Upstream 5'-TGATTGGCGGCTAGGACTTAGG-3' Downstream 5'-TAAGCATGCACACATCGCACA-3'	131
NPHS2	Upstream 5'-TCCGTCTCCAGACCTTGGAAATAC-3' Downstream 5'-TGGATGGCTTTGGACACATGA-3'	145
β-actin	Upstream 5'-GGAGATTACTGCCCTGGCTCCTA-3' Downstream 5'-GACTCATCGTACTCCTGCTTGCTG-3'	150

Table II. Comparison of urine protein quantitation in each group ($\bar{x}\pm s$, mg/24 h).

Group	Control (n=8)	Nephropathy (n=8)	MP+CsA+ MMF (n=8)	MP+FK506+CTX (n=8)	MP+CsA+Rapa (n=8)
0 day	10.32±1.95	11.02±1.67	11.10±2.34	10.29±2.03	10.10±1.53
2 week	11.28±1.40	168.11±13.09	162.04±14.27	159.63±14.73	167.31±13.90
4 week	12.18±1.38	276.41±11.12**	279.81±13.57	285.91±10.95	280.21±14.38
8 week	12.98±2.07	355.76±14.10**	158.67±12.71*	161.83±14.73*	160.64±13.72*
12 week	13.56±2.70	338.92±15.87**	131.29±15.85*	137.18±14.27*	126.30±14.65*

**Compare with control group, $p<0.01$, *Compared with nephropathy group, $p<0.05$.

followed by post-hoc test (Least Significant Difference). The Student-Newman-Keuls (SNK) test was used for comparisons among groups.

Results

Changes in Urine Protein

The 24-h urine protein amounts in the groups are shown in Table II. There were no significant differences for urine protein in the control group at 0, 2, 4, 8, and 12 weeks ($p>0.05$). There were remarkable differences between the nephropathy group and the control group at 2, 4, 8, and 12 weeks ($p<0.01$). There were significant differences in urine protein between the treatment group and the nephropathy group at 8 and 12 weeks by analysis of variance ($p<0.05$). The nephropathy group showed significant differences from the three treatment groups by the SNK test ($p<0.05$). The urine protein in the MP+FK506+Rapa group was lower than that in the other two treatment groups at the end of week 12, but the difference was not significant ($p>0.05$).

Biochemical Parameters

The data for the biochemical parameters are shown in Table III. Several biochemical parameters in the nephropathy group showed significant differences from the control group at 12 weeks, including TP, ALB, TG, and Chol ($p<0.01$). There were significant differences between treatment groups and nephropathy group at 12 weeks by analysis of variance ($p<0.05$). After analysis by the SNK test, nephropathy group showed significant differences from three treatment groups ($p<0.05$). The levels of TP and ALB in treatment groups were significantly higher than those in nephropathy group ($p<0.05$). The levels of TG and Chol in the treatment groups were all significantly lower than those in nephropathy group ($p<0.05$). However, no significant differences were found among treatment groups ($p>0.05$). The levels of BUN and Scr had no significant differences among control group, nephropathy group, and treatment groups ($p>0.05$).

Pathological Changes

The pathological findings are shown in Figure 1. The structures in the renal tissues within the control group appeared normal under light microscopy. Pathological changes were observed in nephropathy group as follows: focal glomerular sclerosis, mesangial cell hyperplasia, podocyte hyperplasia, and small capillaries in a hardened area; increased thickness of glomerular and tubular basement membranes; multiple tubular atrophy, flaky dilatation, and flaking of bristles in renal tubules; denatured protein particles in epithelial cells; and protein casts in the lumen. The pathological changes in the treatment groups were significantly reduced compared with nephropathy group.

Serum Levels of MMP-2, MMP-9, and TGF-β1 Detected by Double-Antibody Sandwich ELISA

The serum levels of MMP-2, MMP-9, and TGF-β1 are shown in Table IV. The MMP-2, MMP-9, and TGF-β1 levels in nephropathy group showed significant differences from control group at 12 weeks ($p<0.01$). The MMP-2 and MMP-9 levels in nephropathy group were significantly reduced compared with control group ($p<0.01$). The MMP-2, MMP-9, and TGF-β1 levels in treatment groups differed significantly from nephropathy group at 12 weeks ($p<0.01$). The MMP-2 and MMP-9 levels in treatment groups were significantly increased compared with nephropathy group ($p<0.05$). The MMP-2 level in MP+CsA+Rapa group was significantly increased compared with the other two treatment groups ($p<0.05$). There was no significant difference between MP+CsA+MMF group

Table III. The changes of biochemical indexes at the end of 12 week ($\bar{x}$±s).

Group	TP (g/L)	ALB (g/L)	TG (mmol/L)	Chol (mmol/L)	BUN (mmol/L)	Scr (umol/L)
Control (n=8)	63.76±3.98	28.78±1.54	0.59±0.20	1.32±0.19	7.83±1.75	55.56±3.97
Nephropathy (n=8)	40.01±3.11$^{\#}$	12.97±1.45$^{\#}$	7.17±1.68$^{\#}$	9.17±1.34$^{\#}$	7.58±1.56	60.21±5.52
MP+CsA+ MMF (n=8)	60.09±3.58$^{\Delta}$	20.13±2.71$^{\Delta}$	4.62±0.72$^{\Delta}$	5.01±0.94$^{\Delta}$	8.51±0.67	57.09±5.78
MP+FK506+ CTX (n=8)	55.17±5.17$^{\Delta}$	19.43±2.03$^{\Delta}$	5.16±0.55$^{\Delta}$	5.55±0.82$^{\Delta}$	8.01±1.43	59.01±2.11
MP+CsA+Rapa (n=8)	56.25±4.36$^{\Delta}$	19.91±1.82$^{\Delta}$	4.18±0.45$^{\Delta}$	6.17±0.94$^{\Delta}$	7.21±1.54	56.92±3.26

$^{\#}$Compared with control group, $p<0.01$, $^{\Delta}$Compared with nephropathy group, $p<0.05$.

Table IV. The expression levels of MMP-2, MMP-9 and TGF-β1 in serum.

Group	Control (n=8)	Nephropathy (n=8)	MP+CsA+MMF (n=8)	MP+FK506+CTX (n=8)	MP+CsA+Rapa (n=8)
MMP-2 (ng/mL)	175.34±5.23	80.71±1.73**	110.90±3.47*	102.17±3.17*	143.36±5.45$^{*\Delta}$
MMP-9 (ng/mL)	79.61±2.18	32.51±1.07**	58.81±1.50*	53.74±0.92*	63.65±1.34*
TGF-β1 (pg/mL)	161.72±6.91	363.02±5.54**	287.20±3.50*	279.35±4.05*	210.63±5.51$^{*\Delta}$

**Compared with control group, $p<0.01$, *Compared with nephropathy group, $p<0.05$, $^{\Delta}$Compared with MP+CsA+MMF and MP+FK506+CTX group, $p<0.05$.

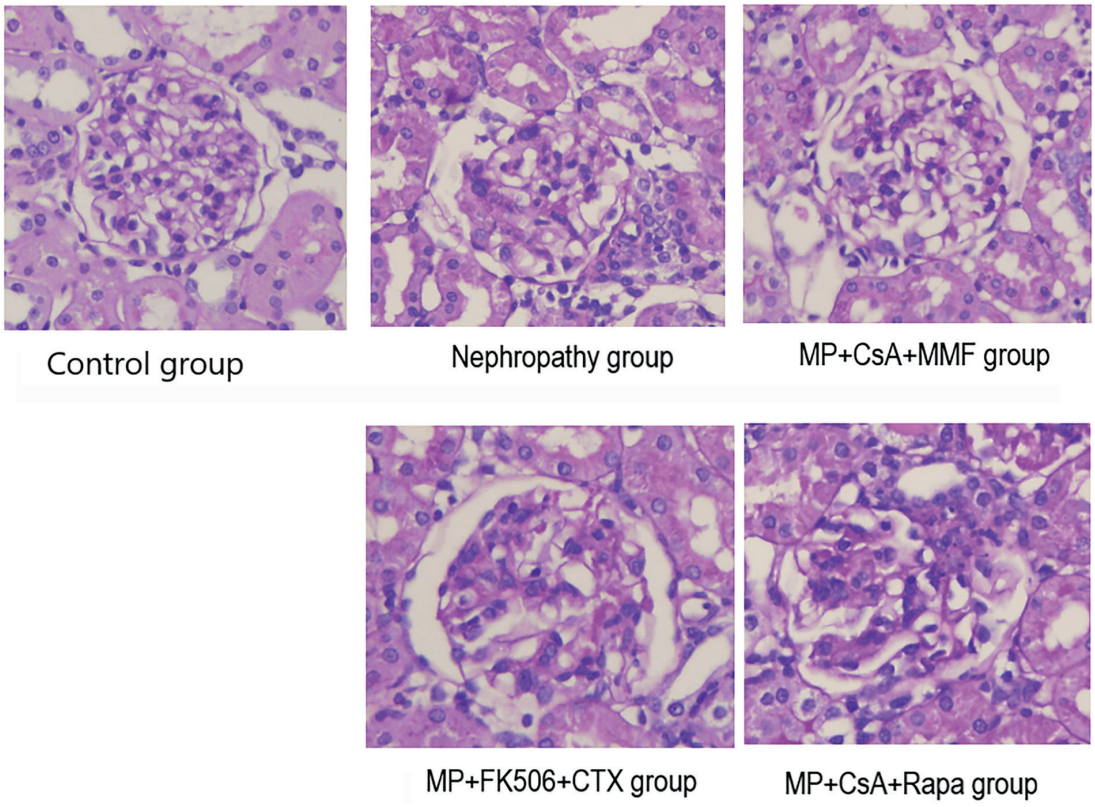

Figure 1. Pathological findings for renal tissues in the groups (magnification ×400).

and MP+FK506+CTX group ($p>0.05$). The MMP-9 level in MP+CsA+Rapa group was increased compared with the other two treatment groups, but the differences were not significant ($p>0.05$). The TGF-β1 level in nephropathy group was significantly increased compared with control group ($p<0.01$) and significantly decreased compared with treatment groups ($p<0.05$). The TGF-β1 lev-

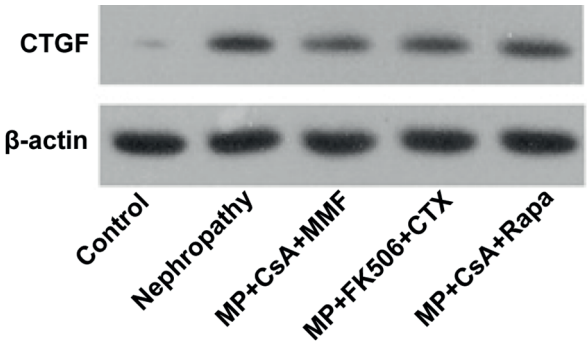

Figure 2. Expression levels of CTGF are significantly changed in the nephropathy and treatment groups.

el in MP+CsA+Rapa group was significantly decreased compared with the other two treatment groups ($p<0.05$), while no significant difference was found between MP+CsA+MMF group and MP+FK506+CTX group ($p>0.05$).

CTGF Expression Levels in Renal Tissues Detected by Western Blotting

The CTGF expression levels are shown in Figure 2. The CTGF expression level in renal tissues differed significantly between nephropathy group and control group ($p<0.01$) and between treatment groups and nephropathy group ($p<0.05$) at 12 weeks. The CTGF expression level in the MP+CsA+Rapa group was significantly lower than those in the other treatment groups by the SNK test ($p<0.05$). There was no significant difference between MP+CsA+MMF group and MP+FK506+CTX group ($p>0.05$).

Expression Levels of Nephrin and Podocin Detected by Immunohistochemistry

The expression levels of nephrin and podocin are shown in Figure 3, Figure 4, and Table V. At 12 weeks, the nephrin and podocin expression levels showed significant differences between nephropathy group and control group ($p<0.01$) and between treatment groups and nephropathy group ($p<0.05$). There were no significant differences between treatment groups based on the SNK test ($p>0.05$). Nephrin and podocin were uniformly and linearly distributed along the glomerular capillaries and highly expressed in control group. In nephropathy group, nephrin and podocin were unevenly distributed in the glomerulus, the staining intensity was weakened, and the linear distribution in some areas disappeared and became a mass. The expression levels of nephrin and podocin were restored in treatment groups.

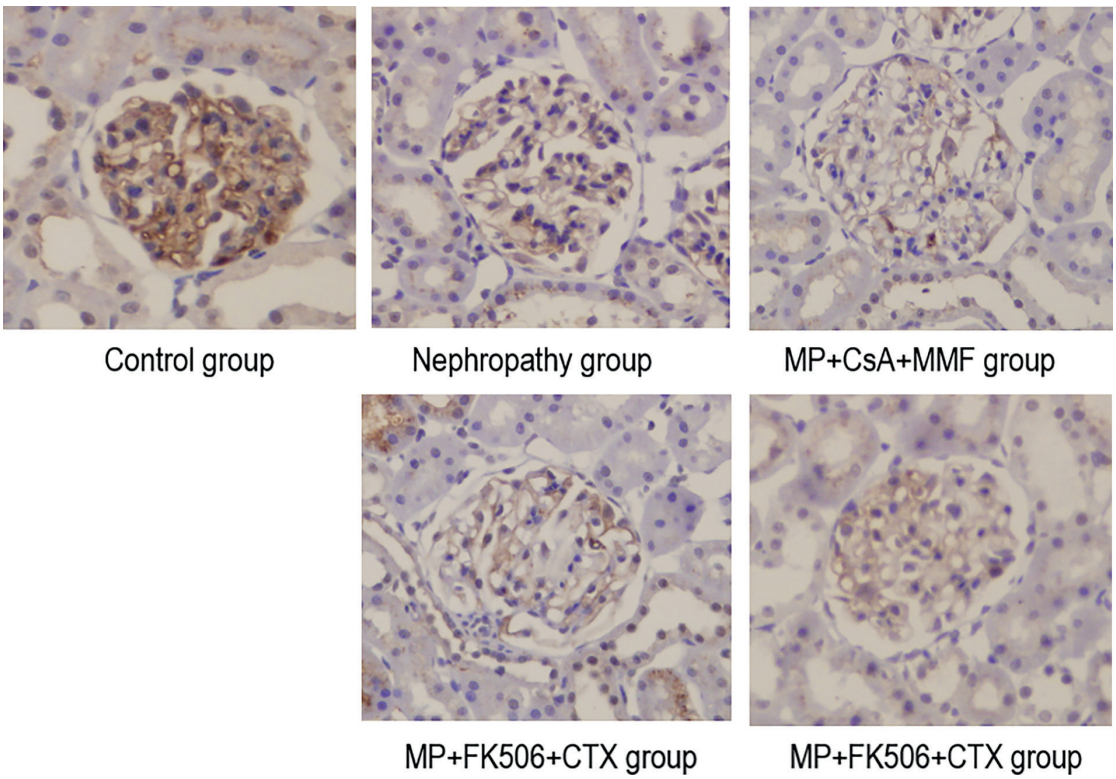

Figure 3. Immunohistochemical staining of nephrin in renal tissues (magnification×400).

Table V. The comparisons of nephrin and podocin in each group by immunohistochemistry.

Group	Control (n=8)	Nephropathy (n=8)	MP+CsA+MMF (n=8)	MP+FK506+CTX (n=8)	MP+CsA+Rapa (n=8)
Nephrin	0.595±0.043	0.252±0.037**	0.451±0.022*	0.479±0.049*	0.483±0.054*
Podocin	0.628±0.037	0.275±0.057**	0.405±0.061*	0.431±0.078*	0.475±0.068*

**Compared with control group, $p<0.01$, *Compared with nephropathy group, $p<0.05$.

Nephrin and Podocin mRNA Levels in Renal Tissues in Each Group

The mRNA levels of nephrin and podocin are shown in Tables VI and VII. At 12 weeks, the nephrin and podocin mRNA levels differed significantly between nephropathy group and control group ($p<0.01$) and between treatment groups and nephropathy group ($p<0.05$) by analysis of variance. There were no significant differences between treatment groups by the SNK test ($p>0.05$). The nephrin and podocin mRNA levels in nephropathy group were significantly decreased compared with control group ($p<0.01$), and the levels in treatment groups were significantly increased compared with nephropathy group.

Discussion

Nephrotic syndrome is a long-term disease characterized by recurrence and is difficult to cure. It has been a challenge for doctors and patients for a long time. With the rapid development of science and in-depth studies on the pathological mechanisms of nephrotic syndrome, more efficient and safer treatments and drugs are emerging. The immune mechanism for glomerular disease is complex, and includes T cell and B cell activation, autoantibody formation, and immune complex formation[2,3]. Therefore, treatment with a single drug has low efficiency. Multi-target application of immunosuppressive agents is a new

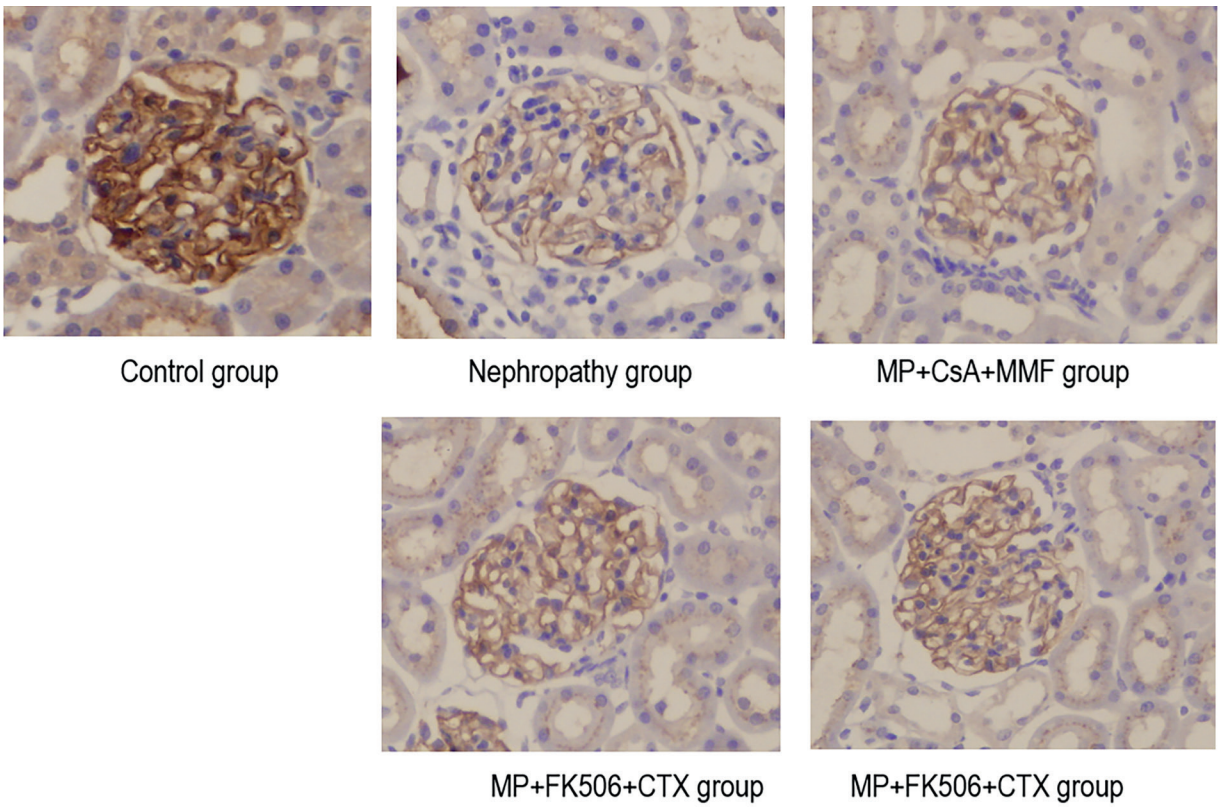

Figure 4. Immunohistochemical staining of podocin in renal tissues (magnification×400).

Table VI. The relative changes of nephrin mRNA level in each group at the end of 12 week.

Group	ΔCt	ΔΔCt	Multiple ($2^{-\Delta\Delta Ct}$)
Control (n=8)	8.173±0.1140	0	1
Nephropathy (n=8)	14.123±0.1273*	5.950	0.016
MP+CsA+MMF (n=8)	10.138±0.8731*#	1.965	0.256
MP+FK506+CTX (n=8)	11.001±0.4930*#	2.828	0.141
MP+CsA+Rapa (n=8)	10.517±0.5201*#	2.344	0.197

*Compared with control group, $p<0.01$, #Compared with nephropathy group, $p<0.01$.

Table VII. The relative changes of podocin mRNA level in each group at the end of 12 week.

Group	ΔCt	ΔΔCt	Multiple ($2^{-\Delta\Delta Ct}$)
Control (n=8)	10.438±0.6526	0	1
Nephropathy (n=8)	14.311±0.1928*	3.873	0.068
MP+CsA+MMF (n=8)	12.142±0.3526*#	1.704	0.307
MP+FK506+CTX (n=8)	12.651±0.1935*#	2.213	0.431
MP+CsA+Rapa (n=8)	12.917±0.3473*#	2.479	0.358

*Compared with control group, $p<0.01$, # Compared with nephropathy group, $p<0.01$.

direction for the treatment of renal disease[4]. However, strategies for how to properly apply various immunosuppressive agents to reduce their toxicity and side effects remain to be explored and established.

Selection of Sequential and Combined Treatments Based on the Cell Cycle

CCSA and/or cell cycle specific and/or non-specific agents (CCNSA) were combined for sequential treatment, and small doses of hormones were supplemented. CsA and MMF are specific drugs for the cell cycle. CsA suppresses immune responses by inhibiting T cell activation in G0-G1 phase, while the active ingredient of MMF is mycophenolic acid, which acts on the late stage of lymphocyte proliferation (G1-S phase). FK506 (tacrolimus) is a novel calmodulin inhibitor with a similar mechanism to CsA. FK506 selectively inhibits the production of interleukin-2 by inhibiting the activity of calmodulin, as well as inhibiting the proliferation of lymphocytes by blocking lymphocytes in G0-G1 phase[5,6]. Rapamycin (Rapa) is a novel macrolide immunosuppressive agent. After introduction to cells, Rapa binds to FK-binding protein 12 (FK-BP12) to form a complex with mammalian target of rapamycin (mTOR)[7-9], thereby preventing cell cycle transformation from G1 phase to S phase and initiation of protein translation. This leads to an anti-proliferative effect and blocks G1-S phase progression of T cells and other cell types. CTX is a bifunctional alkylating agent and non-specific drug for the cell cycle. CTX reduces the amounts of T cells, B cells, and antibody production, inhibits proliferation of lymphocytes, and kills cells in various stages of the cell cycle. The working principles for treatment groups were as follows. For hormone+CsA+MMF treatment group, the initial maintenance treatment with CsA arrests most of the T cells in G0-G1 phase for 1 week, and the secondary treatment with MMF for 1 week arrests most T cells and B cells in G1-S phase. The sequential and combined use of drugs for different cell cycle phases exerts blocking and killing effects for each immunosuppressive drug and restores drug-induced injuries during the intermittent interval. For hormone+FK506+CTX treatment group, the initial maintenance treatment with FK506 arrests most of the lymphocytes in G0-G1 phase for 1 week, and the secondary treatment with the non-specific cell cycle drug CTX for 1 week kill the cells in quiescent G0 phase and various proliferating stages (including S phase). For hormone+CsA+Rapa treatment group, the principle of the effects is similar to that for hormone+CsA+MMF treatment group. Specifically, the initial maintenance treatment with CsA for 1 week arrests most of the T cells

in G0-G1 phase, and the secondary treatment with MMF for 1 week arrests most of the T cells and B cells in G1-S phase. The sequential and combined use of drugs for different cell cycle phases exerts blocking and killing effects of CsA and Rapa, and restores the drug-induced injuries in the intermittent interval.

Therapeutic Effects for Adriamycin-Induced Nephropathy in Rats

The adriamycin-induced nephropathy model is recognized as a model for improved simulation of glomerular disease. The model exhibits minimal pathological nephropathy in a short time period. Focal segmental glomerulosclerosis is induced by increased injection dose, longer observation time, or repeated injections[10]. Two injections were used in the present study to create a stronger pathology and more stable research model. The following phenotypes were observed in the nephropathy group at the end of week 12: extensive fusion of glomerular foot processes, proliferation of podocytes and mesangial cells, increased width of mesangial area, increased thickness of basement membrane, interstitial fibrosis, glomerular segmental sclerosis, narrowed capillaries in hardened area, multiple tubular atrophy, flaky dilatation and flaking of bristles in renal tubules, denatured protein particles in epithelial cells, and protein casts in the lumen. The three treatment plans all achieved satisfactory therapeutic effects in this adriamycin-induced nephropathy model in rats.

The therapeutic effects of the three treatment plans for pathology were examined by 24 h urine protein quantification and blood biochemical parameters in the adriamycin-induced nephropathy rat model. After application of the three treatments to adriamycin-induced nephropathy rats, the renal pathology was significantly reduced compared with the nephropathy group. The 24 h urine protein was significantly reduced in three treatment groups, and the urine protein amount in MP+CsA+Rapa group was lower than that in the other two treatment groups at the end of week 12, but without a significant difference. The three treatment plans significantly increased the levels of TP and ALB, and decreased the levels of TG and Chol. The levels of BUN and Scr showed no significant changes in the three treatment groups. These results indicate that therapeutic effects were achieved in all three treatment groups, and no adverse reactions, such as nephrotoxicity, were observed.

The therapeutic Effects of the Three Treatment Plans for Podocyte-Related Factors Nephrin and Podocin in Adriamycin-Induced Nephropathy Rats

Podocytes are highly specific and terminally differentiated cells with extremely complicated cell structures and diverse biological functions. Studies showed that nephrin and podocin on the membrane of glomerular podocytes play key roles in the production of proteinuria. They form a slit membrane complex (termed slit diaphragm), which creates a fissure membrane by connecting the foot processes and is the final barrier to protein filtration for glomerular macromolecules[11,12]. Further studies showed that nephrin and podocin play important roles in slit diaphragm integration and integrity maintenance for the glomerular filtration barrier. When the protein or amino acid composition of nephrin and podocin is changed, the membrane pores disappear and a large amount of proteinuria will occur[13,14]. Ahn et al[15] described high expression levels of nephrin and podocin in the early stage of proteinuria in rats with adriamycin-induced nephropathy. Although the expression levels of nephrin and podocin were increased, the distributions of the molecules were abnormal by immunofluorescence staining. These observations indicate that the increased molecules in podocytes are not correctly located in the membrane, and instead are likely to be stored in the nucleus or other organelles like the endoplasmic reticulum during protein secretion. As a result, the increased podocyte-related molecules cannot exert their normal biological functions, leading to progressive exacerbation of proteinuria in rats with adriamycin-induced nephropathy. The increased expression may be a compensatory response to early injury of podocytes, and the expression levels of nephrin and podocin gradually decrease with gradual progression of the nephropathic pathology.

The protein and mRNA levels of nephrin and podocin were significantly decreased in adriamycin-induced nephropathy rats, as evaluated by immunohistochemistry and real-time quantitative RT-PCR in the present study. Their distributions changed in the glomerulus, producing granular and irregular distributions along the glomerular basement membrane. The probable mechanism is that podocyte molecules maintain their structure and function through compensatory synthetic proteins. As the disease progresses, the expression of podocyte-related molecules enters the decompensation phase, resulting in progressive de-

creases in the expression of nephrin and podocin. The expression and distribution of nephrin and podocin were restored in the treatment groups, demonstrating that the three treatment plans could protect the glomerular filtration barrier integrity and reduce production of proteinuria. The possible reasons are as follows. When the natural cells in the kidney are damaged by sustained pathological stimulation, the podocytes maintain their structure and function through limited compensatory responses. However, immunosuppressive agents also block the cell cycle of the intrinsic renal cells[16-18]. This might weaken the compensatory capacity of podocytes and aggravate their damage. The sequential treatments not only have anti-inflammatory and immunosuppressive effects, but also enable recovery of renal cells in the intermittent period of drug use.

The effects of the Three Treatment Plans on Renal Fibrosis-Related Factors in Adriamycin-Induced Nephropathy Rats

Renal interstitial fibrosis is a common pathway for the development of chronic or progressive renal disease to end-stage renal disease. The characteristic pathological changes are as follows: infiltration of large numbers of inflammatory cells, fibroblast activation, and tubular atrophy. These changes lead to excessive accumulation of extracellular matrix (ECM), resulting in replacement of renal structures and even insufficiency or loss of function in severe cases. Large amounts of clinical practice and experimental data have shown that the severity of renal interstitial fibrosis is an important factor in determining the prognosis of renal disease[19].

Factors that promote renal fibrosis include vasoactive factors, growth factors, inflammatory chemokines, and adhesion molecules, among which the effects of TGF-β and CTGF are the most prominent. TGF-β belongs to the TGF superfamily, and its role in renal interstitial fibrosis is mainly as follows. First, TGF-β directly promotes the expression of types I, II, III, IV, and V collagen (mainly type I collagen), proteoglycan, and fibronectin for the ECM, inhibits the activity of MMPs and enhances the activity of tissue inhibitors of matrix metalloproteinase (TIMPs)[20]. Second, TGF-β inhibits the expression of MMPs. Many reports have shown that MMPs like MMP-2 and MMP-9 play key roles in ECM degradation, and are regulated at many levels to affect ECM degradation[21]. CTGF is a cysteine-rich growth factor that belongs to the CCN family[22]. It is an early gene product induced by TGF-β and related to the biological effects of TGF-β. High CTGF mRNA expression is found in renal biopsy specimens from patients with FSGS, crescentic nephritis, IgA nephropathy, and diabetic nephropathy[23,24]. CTGF expression is mainly increased in glomerular mesangial areas and tubular interstitial lesions with cell proliferation and ECM synthesis[25]. Studies have demonstrated that CTGF induces phosphorylation of LRP-1 tyrosine, activates the ERK1/2 signaling pathway, and promotes TGF-β1-induced differentiation of renal interstitial fibroblasts into myofibroblasts. These actions lead to overexpression of ECM and occurrence of interstitial fibrosis.

TGF-β and MMPs are the key factors that influence ECM accumulation and degradation. The balance of MMPs/TIMPs has a crucial role in the formation of glomerular sclerosis. Expression of TGF-β1 is related to an imbalance of MMPs/TIMPs[26]. Furthermore, TGF-β induces transdifferentiation of renal tubular epithelial cells to mesenchymal cells, enhances proliferation of renal interstitial fibroblasts[27], and promotes apoptosis of renal tubular epithelial cells. Finally, TGF-β1 induces apoptosis of vascular endothelial cells, reduces the number of tubular capillaries, inhibits regeneration of perivascular capillaries, and accelerates progression of renal interstitial fibrosis[28]. The serum levels of TGF-β1, MMP-2, and MMP-9 and the expression of CTGF in renal tissues were evaluated in adriamycin-induced nephropathy rats. In the three treatment groups, the serum level of TGF-β1 was decreased, the serum levels of MMP-2 and MMP-9 were increased, and the expression of CTGF in renal tissues was reduced. The effects of the hormone+CsA+Rapa treatment were especially remarkable among the three treatment groups. On the one hand, sequential use of immunosuppressive agents for the cell cycle can directly reduce the expression of TGF-β1 and its downstream factor, CTGF; On the other hand, reducing TGF-β1 can promote the expression of MMP-2 and MMP-9, thereby reducing the deposition of ECM. These effects improve the prognosis of CKD and delay the occurrence of end-stage renal disease.

In the established rat model of adriamycin-induced nephropathy, urine protein, blood biochemical parameters, podocyte injury-related factors, and renal fibrosis-related factors were evaluated. The results indicated that the three sequential treatments (hormone+CsA+MMF, hormone+FK506+CTX, and hormone+CsA+Rapa) according to the cell cy-

cle are feasible and provide novel ideas for the treatment of glomerular diseases.

Conclusions

This study suggests that, for the treatment of refractory nephrotic syndrome, the use of immunosuppressive agents acting at different target sites and different phases of the cell cycle could be useful when combined use of immunosuppressive agents is required. The immunosuppressive agents are sequentially combined according to the cell cycle, to avoid simultaneous use of multiple immunosuppressive agents that act on the same sites or the same phases of the cell cycle. By using sequential treatment, the therapeutic effect of each immunosuppressive agent is fully utilized and the adverse reactions caused by excessive inhibition of immunity are avoided.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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