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Original Article

Changing trend and significance of serum IL-2 and IL-4 levels in rats with nephrotic syndrome

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Abstract: Objective: To investigate the relation between nephrotic syndrome (NS) and expression of interleukin-2 (IL-2) and interleukin-4 (IL-4) in serum of a NS rat model. Methods: A total of 40 healthy male Wistar rats were modeled and among these, 30 were randomly selected as the observation group according to a random number table. The NS model was established by one-time jugular vein injection of puromycin aminonucleoside. The remaining 10 rats underwent sham operation and served as the control group. After the model was established, peripheral blood was collected from all rats in both groups via the orbital venous plexus on day 1, day 7, and day 14. Serum levels of IL-2 and IL-4 levels were determined by enzyme-linked immunosorbent assay, and serum albumin and renal function index were measured by automatic biochemical analyzer. Prior to sacrificing the animals, urine was collected for 24 hours, and the biuret method was employed to determine the 24-hour urinary protein excretion rate. Results: In the observation group, typical NS symptoms, including proteinuria and edema, were observed 14 days after surgery. In 27 rats, the NS model was successfully established, and 3 rats died. The success rate was 90%. In the control group, 1 rat died 14 days after sham surgery, and the surgical success rate was 90%. The serum levels of IL-4 and creatinine levels on day 1, day 7, and day 14 of the observation group were significantly higher compared to those of the control group (all $P<0.001$). The 24-hour urinary protein excretion rates on day 7 and day 14 of the observation group were significantly higher than those of the control group (both $P<0.001$). Moreover, the levels of IL-2 and serum albumin of the observation group were lower than those of the control group (both $P<0.001$). In addition, on day 1 and day 7, the levels of blood urea nitrogen in the observation group were significantly higher than those in the control group (both $P<0.05$). Conclusion: The IL-2 level in the serum of NS rats decreased, whereas the IL-4 level increased, which might be related to the occurrence and development of NS.

Keywords: Interleukin-2, interleukin-4, nephrotic syndrome, rat, renal function

Introduction

Nephrotic syndrome (NS) includes primary NS and secondary NS. Among all NS patients, approximately 2/3 of adults suffer from primary NS, whereas 1/3 suffer from secondary NS. In addition, approximately 90% of pediatric NS patients are diagnosed with primary NS, and 10% suffer from secondary NS [1, 2]. Primary NS mostly occurs in people of 40 years old and younger, and is more prevalent in men than women [3]. NS can significantly decrease levels of IgG, leading to decreased immune function and concurrent infection; patients with severe NS may develop renal insufficiency and uremia, which severely endangers the health of patients, and even leads to death [4, 5]. Previous studies have shown that improving the immune function of patients is an important approach to prevent and treat NS [6].

Interleukin-2 (IL-2) has an immunoprotective effect and stimulates expression of immune cells and immune factors and enhances the immune response [7]. Interleukin-4 (IL-4) is a cytokine that enhances secretion of B cells and regulates humoral and adaptive immunity [8]. In several studies, it has been shown that expression of IL-2 and IL-4 increased in the peripheral blood of patients with poor immune function [9, 10]. However, in recent years, little research has been performed on the relation between IL-2, IL-4, and NS.

We hypothesized that IL-2 and IL-4 may be related to the development and progression of NS.

Therefore, we established a rat NS model to investigate changes of expression of IL-2 and IL-4, urinary protein, serum albumin, and renal function, and show the significance of IL-2 and IL-4 in the development of NS.

Materials and methods

Experimental animals

This study was approved by the Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Specific-pathogen free (SPF) healthy male Wistar rats (aged 9-11 weeks, weighing 221-350 g), were purchased from Wuhan Hualianke Biological Technology Co., Ltd. (Wuhan, China) and were fed a Shuke-Beta SPF-grade rat chow (Jiangsu Xietong Organism Co., Ltd., Jiangsu, China). Rats were housed at room temperature (24±3°C). The ammonia concentration did not exceed 20 ppm, airflow velocity was (18±5 cm/s), and ventilation frequency was 12±3 time/h. Additional housing conditions were as follows: humidity of 50-70%, fluorescent lighting, free access to food and drinking water (sterile disinfectant), feeding boxes were changed once to twice a week, and water bottles were changed 3-4 times a week.

Establishing an animal model of NS

For general anesthesia, rats were given an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Satisfactory anesthesia was followed by jugular vein catheterization. Rats in the observation group were given a one-time slow injection with puromycin aminonucleoside (Shanghai Sanshu Biotechnology Co., Ltd., Shanghai, China) in physiological saline (40 mg/kg), and rats in the control group were given a similar volume of physiological saline solution. The 24-hour urinary protein excretion before and after modeling was recorded.

Determination of urinary protein by biuret method

Urine from all rats in metabolic cages was collected for 24 hours on a daily basis prior to euthanasia and analyzed by biuret method (Shanghai Jingke Chemical Technology Co., Ltd., Shanghai, China).

Preparation of the standard curve: Twelve test tubes were divided into two groups. The standard protein solution in volumes of 0, 0.2, 0.4, 0.6, 0.8, 1.0 mL was added and water was added to make a total volume of 1 mL in each tube. Then, 4 mL of biuret reagent was added. The solution was well-mixed and placed at room temperature for 30 minutes, and colorimetric measurement was performed at 540 nm. A solution that did not contain protein solution was used as a blank. The average values of two groups of measurements were taken and the protein content was used as the horizontal coordinate and the light absorption value as the longitudinal coordinate, and both were used to create the standard curve.

Sample determination: The protein concentration of unknown samples was determined using similar conditions as mentioned above (1 mL sample + 4 mL biuret reagent). The sample concentration should not exceed 10 mg/mL.

Determination of venous blood biochemical indicators

From the two groups of rats, blood was collected from the orbital venous plexus on day 1, day 7, and day 14 after the NS model was established in the observation group. The Becker Olympus AU-5800 automatic biochemical analyzer (Beckman Olympus, USA) was used to determine serum levels of albumin (Alb), blood urea nitrogen (BUN), and creatinine (Cr).

Determination of serum IL-2 and IL-4 levels by enzyme-linked immunosorbent assay

Serum levels of IL-2 and IL-4 were determined by enzyme-linked immunosorbent assay (ELISA). Assay kits were purchased from Shanghai Jingkang Biological Engineering Co., Ltd. (China) and experimental procedures were performed following the manufacturer's guidelines provided with the kit. A total of 50 μL of the sample to be examined was added to each well of a microplate. Two positive and two negative control wells were included. One drop of positive control was added to each well, and 1 well of blank control was included. One drop of enzyme conjugate was added per well and mixed well, then the plate was sealed and incubated at 37°C for 30 minutes. From each well, the liquid was discarded and washing solution was added to the wells. After standing for 5 seconds, the plate was spun and dried at room temperature. This was repeated 5 times, and the plate was patted dry. Per well, 1 drop of chromogenic

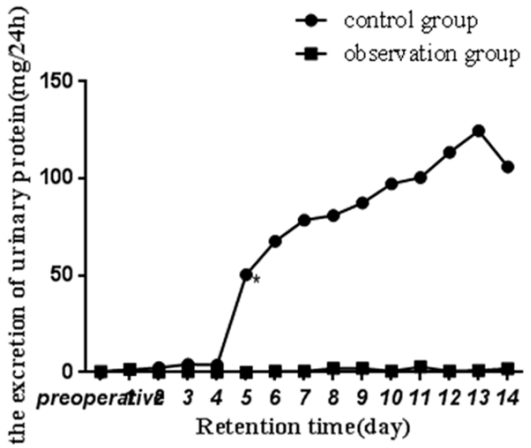

Figure 1. The excretion rate of urinary protein at 24 hours in the two groups before and after operation. Before and after surgery, urine was collected from all rats 24 hours later. Protein levels in urine were determined by the biuret method. From the fifth day, compared with control group, *P<0.05.

agent A solution and 1 drop of chromogenic agent B solution were added, the plate was mixed well, sealed, and incubated at 37°C for 15 minutes. Per well, 1 drop of stop solution was added and mixed thoroughly. Using a microplate reader, a wavelength of 450 nm was used, and a zero calibration was performed using the blank, and the OD was read of each well.

Statistical analysis

In this study, SPSS 19.0 was used. The rate data were compared using the X^2 test. Measurement data are expressed as mean ± standard deviation ($\bar{x}$ ± sd). Between two groups, the non-parametric K-S test was used for data that did not meet a normal distribution. The t-test was used for the data that were normally distributed. P<0.05 is considered statistically significant.

Results

Establishing an animal model of NS

A total of 40 healthy male Wistar rats were modeled among which 30 rats were selected by a random number table method as the observation group to establish NS models. After 14 days, typical NS symptoms were observed, including proteinuria and edema. In this study, 27 models were successfully established, and 3 rats died of insufficient tolerance, leading to a success rate of 90%. The remaining 10 rats underwent sham-surgery and served as a control group. After 14 days, one rat died of insufficient tolerance and the success rate was 90%.

The 24-hour urinary protein excretion rate before and after surgery in both groups

Prior to surgery, no differences were observed in the 24-hour urinary protein excretion rate between the two groups (P>0.05). On day 5 after surgery, proteinuria was observed in the observation group, which peaked on day 13. No significant changes in 24-hour urinary protein excretion rate were observed in the control group (**Figure 1**). Moreover, on day 14, the 24-hour urinary protein excretion rate in the observation group started to decrease, and the excretion rate on day 14 was higher compared to that on day 1 (P<0.05). No differences were found when compared with day 7 (P>0.05), and the excretion rate on day 7 was higher compared to that on day 1 (P<0.05). The 24-hour urinary protein excretion rate of the control group did not significantly change (P>0.05). The 24-hour urinary protein excretion rates of rats in the observation group on day 7 and day 14 were higher compared to those of the control group (both P<0.05).

Changes in serum albumin levels in NS rats

The results show that serum Alb levels of rats in the observation group on day 1, day 7, and day 14 were lower compared to those in the control group (all P<0.001). In the observation group, serum Alb levels gradually decreased with time, and significant differences were found in serum Alb levels between every two time points within group (both P<0.001). In the control group, no differences were observed in serum Alb levels between every two time points (both P>0.05) as shown in **Table 1**.

Changes in renal function index in NS rats

The results showed that serum Cr levels in rats in the observation group on day 1, day 7, and day 14 were higher when compared to those in the control group (all P<0.001). In addition, BUN levels were higher compared to those in the control group only at day 1 and day 7 (both P<0.05). In rats in the observation group, ser-

Table 1. Serum Alb levels of rats on day 1, 7, and 14 after the NS model was established (g/L)

	Control group	Observation group	Statistic	P
Before operation	49.33±1.69	49.47±1.62	0.632	0.925
Day 1	48.74±1.51	41.55±1.96[a]	20.341	<0.001
Day 7	46.12±1.47	32.29±1.25[a,b]	56.242	<0.001
Day 14	45.33±1.28	26.47±1.35[a,b,c]	74.751	<0.001

Note: Alb, serum albumin; NS, nephrotic syndrome. [a]P<0.001, compared with before operation; [b]P<0.001, compared with day 1; [c]P<0.001, compared with day 7.

Table 2. Changes in renal function index of rats on day 1, 7, and 14 after the NS model was established

	Control group	Observation group	Statistic	P
BUN (mmol/L)				
Before operation	7.76±1.12	7.88±1.09	0.588	0.859
Day 1	7.25±1.03	7.84±1.11[a]	2.856	0.005
Day 7	6.61±0.92	7.13±1.02[a,b]	2.754	0.007
Day 14	6.62±0.87	6.54±1.03[a,b,c]	0.425	0.672
Cr (μmol/L)				
Before operation	57.69±2.33	58.74±2.69	0.822	0.733
Day 1	51.33±2.01	58.66±4.11[a]	14.822	<0.001
Day 7	49.12±1.64	56.28±3.85[a,b]	16.393	<0.001
Day 14	48.50±1.98	55.31±3.23[a,b,c]	15.594	<0.001

Note: BUN, blood urea nitrogen; Cr, creatinine; NS, nephrotic syndrome. [a]P<0.001, compared with the same index before operation; [b]P<0.001, compared with the same index on day 1; [c]P<0.001, compared with the same index on day 7.

Table 3. IL-2 levels of rats on day 1, 7, and 14 after the NS model was established (μg/L)

	Control group	Observation group	Statistic	P
Before operation	4.42±1.11	4.43±1.12	0.554	0.892
Day 1	4.35±1.09	3.28±0.84[a]	6.342	<0.001
Day 7	4.26±1.11	2.95±0.77[a,b]	8.098	<0.001
Day 14	4.12±0.96	2.74±0.47[a,b,c]	11.941	<0.001

Note: IL-2, interleukin-2; NS, nephrotic syndrome. [a]P<0.001, compared with before operation; [b]P<0.001, compared with d1; [c]P<0.001, compared with day 7.

Table 4. The IL-4 levels of rats day 1, 7, and 14 after the NS model was established (pg/L)

	Control group	Observation group	Statistic	P
Before operation	1.52±0.33	1.55±0.42	0.612	0.933
Day 1	1.48±0.20	3.49±0.36[a]	32.073	<0.001
Day 7	1.23±0.14	3.58±0.37[a]	37.285	<0.001
Day 14	1.11±0.13	4.48±0.64[a,b,c]	31.342	<0.001

Note: IL-4, interleukin-4; NS, nephrotic syndrome. [a]P<0.001, compared with before operation; [b]P<0.001, compared with day 1; [c]P<0.001, compared with day 7.

um BUN, and Cr levels gradually decreased with time and were both different between every two time points in both groups (all P<0.001) as shown in **Table 2**.

Changes of IL-2 in serum of NS rats

IL-2 levels were determined by ELISA. The results showed that IL-2 levels in rats in the observation group at the three time points were all lower than those in the control group (all P< 0.001). Moreover, in the observation group, the IL-2 level gradually decreased with time, and differences were observed in IL-2 levels between every two time points (all P<0.001). In the control group, no differences were observed in IL-2 levels between every two time points (all P>0.05) as shown in **Table 3**.

Changes in IL-4 in serum of NS rats

IL-4 levels were determined by ELISA. The results showed that IL-4 levels in rats in the observation group were all higher than those in the control group at three time points (all P<0.001). In the observation group, the IL-4 level gradually increased with time. The IL-4 level on day 14 was higher than that on day 1 and day 7 (P< 0.001), and the IL-4 level on day 7 was not different from that on day 1 (P>0.05). In the control group, no differences were observed in IL-4 levels between every two time points (all P>0.05) as shown in **Table 4**.

Discussion

NS is a multifactorial disease, and the underlying mechanisms have not yet been elucidated [11]. In several studies it has been shown that cytokines play a very important role in NS

[12, 13]. Zhang et al. demonstrated that IL-2 and IL-4 were closely related to the disease severity and prognosis of patients with idiopathic NS [14]. We established an NS Wistar rat model to investigate the changes in the expression of IL-2 and IL-4 in NS and their significance.

The results of this study indicate that the serum Alb level in rats in the observation group gradually decreases, thereby indicating that the filtering ability of the glomerulus declined. However, the decrease in 24-hour urinary protein excretion rate in rats in the observation group may be associated with the worsening of the NS condition in rats, which affected protein synthesis, and may be related to the modeling approach used in our study. The stability of rat NS was not high, which was also reflected by the decrease in serum BUN and Cr levels in rats in the observation group. In future experiments, we will explore the improved method of rat NS model replication and hope to overcome this shortcoming in future experiments.

The results of this study showed that IL-2 expression in serum of NS rats was lower compared to that of sham-operated rats, whereas expression of IL-4 was increased in the serum of NS rats. IL-2 is mainly secreted by T lymphocytes, while IL-4 is mainly secreted by T helper (Th2) lymphocytes [15, 16]. Immune dysfunction plays an important role in the pathogenesis of NS [17]. Pereira et al. showed that the number of T lymphocytes in patients with NS decreased, regulatory T lymphocytes decreased, and when the disease progressed further, regulatory T lymphocytes further declined. This resulted in inhibition of T lymphocyte proliferation and IL-2 secretion [18]. In this study, we showed that 1 day after successful modeling, the IL-2 level in the serum of rats in the observation group decreased, and this decrease was statistically significant between day 1, day 7, and day 14. The results suggested that, over time, the condition of rats with NS worsened and the number of T-lymphocytes decreased. Therefore, IL-2 levels gradually decreased.

Murphy et al. reported that NS disrupted homeostasis of Th1/Th2 cells and tended to result in a Th2 phenotype, which may be one of the reasons for the increase in IL-4 levels [19]. In our study, serum levels of IL-4 of rats in the observation group were decreased as the condition of NS worsened, which may be related to restoration of renal function in rats to a certain extent [20]. Because of the limited conditions of the study, we could not observe the morphological aspects of rat kidney tissue. The mechanism of IL-2 and IL-4 in the development of NS is still not revealed, and we will further explore this in future experiments.

In summary, serum IL-2 levels in NS rats decreased, whereas IL-4 levels increased, which may be related to the occurrence and development of NS.

Disclosure of conflict of interest

None.

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