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ORIGINAL ARTICLE

Chronic Toxicity of a Novel Recombinant Human Granulocyte Colony-stimulating Factor in Rats[△]

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Key words: chronic toxicity; Sprague-Dawley rat; novel recombinant human granulocyte colony-stimulating factor

Objective To assess the severity and reversibility of the chronic toxicity of a novel recombinant human granulocyte colony-stimulating factor (rhG-CSFa) in rats and the dose-effect relationship.

Methods A total of 100 Sprague-Dawley rats (equal numbers of male and female) were randomly divided into five groups (20 rats in each group): four groups were treated with rhG-CSFa at 500, 100, 10, 1 µg/kg, respectively, and one group was treated with vehicle only to serve as the control. The rats were received subcutaneous injections of rhG-CSFa or vehicle daily for 13 weeks. During the course of the chronic toxicity study, the physical status, body weight, and food consumption were monitored. Half of the rats in each group ($n=10$) were sacrificed after the last rhG-CSFa administration, and the other half were sacrificed at five weeks after the last rhG-CSFa administration. Urinalyses, blood biochemistry, hematological analysis, histopathological examination, and immunological tests were performed for each of the rats.

Results The hematological analyses revealed that the mean white blood cells count, neutrophils count, and neutrophils percentage were increased in male rats at the dose of 10 µg/kg or higher, and these were related with the biological activity of rhG-CSFa. Some small abnormalities were observed in the spleen of a few rats when used highest dose (500 µg/kg, a dosage of 200 folds higher than the normal clinical dosage), but these abnormalities were recovered within 5-week recovery period. No other rhG-CSFa-related abnormalities were observed in this chronic toxicity study.

Conclusion No significant toxicity and immunogenicity are observed with rhG-CSFa administration to rats in the chronic toxicity studies.

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GRANULOCYTE colony-stimulating factor (G-CSF) can promote proliferation, differentiation, and maturation of neutrophilic granulocyte precursors.¹ Recombinant human G-CSF (rhG-CSF) has been successfully used as a therapeutic drug for neutropenia² and mobilization of marrow hematopoietic stem cell³ since 1991. Compared with wild-type rhG-CSF, a novel recombinant human granulocyte colony-stimulating factor (rhG-CSFa), which has four additional amino acids (Met-Arg-Gly-Ser) at its N-terminal, is able to enhance the affinity of rhG-CSFa to bind to the membrane receptors in its target cells. In addition, the cysteine on the 17th position of the rhG-CSFa molecule was site-mutated and replaced by an alanine in order to reduce the possible disulfide bond formation between molecules.⁴ Previous animal studies of rhG-CSFa have shown greater biological activity than wild-type rhG-CSF.⁵ The pharmacokinetic study in rats also indicated that the half life of rhG-CSFa was longer than that of wild-type rhG-CSF.⁶ The following study was performed based on China standard guideline⁷ and other references⁸ to assess the severity and reversibility of the chronic toxicity of rhG-CSFa in rats.

MATERIALS AND METHODS

Animals and housing conditions

A total of 100 Sprague-Dawley rats (equal numbers of male and female, 4-week old, body weights: about 86 g for female and 100 g for male) were obtained from the Experimental Animal Center of Soochow University (Suzhou, China), Certificate no.: SYXK (Su) 2007-0035. Rats were acclimated for 7 days before treated with drug and randomly divided into five groups ($n=20$ each, equal numbers of male and female). Animals were housed in stainless steel cages with 5/cage in a room with a regulated temperature of 18°C-29°C, relative humidity of 40%-70%, and a 12-hour light cycle (illuminance: 150-300 lx), conforming to Laboratory Animal-Requirements of Environment and Housing Facilities (GB14925-2001). Chow and water was available *ad libitum* throughout the study.

rhG-CSFa administration

GMP standard rhG-CSFa was produced and provided by Biopharmagen Co. (Fangzhou, Beihai, China). The protein solution with a concentration of 250 µg/mL contained 10 mmol/L sodium acetate (pH 4.0), 5% mannitol, and 0.004% Tween 80. rhG-CSFa used in the present study was diluted to the appropriate concentrations with PBS buffer. The highest dose used was 500 µg/kg, a dose of 200-fold higher than normal clinical dosage, and the other three

doses used were 100, 10, and 1 µg/kg. The control group was treated with vehicle only. The dose volume was 2 mL/kg and rhG-CSFa was dosed into the subcutis of the dorsal skin once daily for 13 weeks. Half of the rats in each group were sacrificed after the last rhG-CSFa administration, and the remaining animals were allowed a 5-week recovery period without further rhG-CSFa treatment so that the reversibility of any effects noted at the end of the treatment period could be assessed. The urine, blood, and tissues of each rat were collected for further examinations at the end of the treatment period or recovery period.

Routine examinations

The first day and week of dosing were designated as Day 1 and Week 1. During the course of the chronic toxicity study, the physical status was monitored daily, food consumptions were determined weekly. Body weights were taken at Day 1, twice a week during the study, and at gross pathology.

Urinalyses

Urinalyses was conducted using Uritest-500B urine analyzer (URIT, Guangxi, China) and the parameters measured were pH, specific gravity, glucose, occult blood, protein, bilirubin, urobilinogen, ketone body, and white blood cells (WBC).

Blood biochemistry analysis

Comprehensive blood biochemistry analysis was performed on a 7180 automatic analyzer (HITACHI, Japan) using serum isolated from whole blood collected from abdominal aorta. The blood chemistry parameters included total bilirubin, total protein (TP), serum albumin (ALB), albumin/globulin (A/G), aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase (ALP), glucose, urea nitrogen (BUN), creatinine, triglyceride (TG), total cholesterol (CHO), and phosphokinase (CPK).

Hematology analysis

Comprehensive hematology analysis was performed on a MEK-7222K automatic hematology analyzer (NIHON KOHDEN, Japan) using blood collected from abdominal aorta and treated with di-potassium-EDTA. Hematological parameters evaluated included WBC and differential cell counts (neutrophils, lymphocytes, monocyte, eosinophils, basophils), red blood cell counts, hemoglobin concentration, mean cell hemoglobin, mean corpuscular hemoglobin concentration, red blood cell distribution width, plateletcrit (PCT), and mean platelet volume (MPV). Prothrombin time was measured using a TRT4 semi-automatic coagulometer

(Behnk Elektronik, Germany).

Gross pathology, organ weights, and histopathology

All rats were fasted at least 16 hours and subjected to a complete gross pathology after being anesthetized. Absolute organ weights of brain, heart, lung, liver, kidneys, adrenals, thymus, spleen, testes, epididymis, prostate, ovaries, and uterus were determined and the animal organ/body weight ratio was calculated (unit: g/100 g). A full histopathological examination was performed for not only those organs mentioned above, but also additional organs, including pituitary, thyroid, tongue, bone marrow of sternum, spinal marrow, esophagus, submandibular glands, stomach, intestine, pancreas, trachea, thoracic aorta, mammary glands, urinary bladder, dorsal skin, mesenteric lymph nodes, and joint of hind limbs. Tissues were preserved in neutral-buffered 10% formalin 7 days at least, routinely processed and embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

Anti-rhG-CSFa antibody assay

Additional serum samples were collected at the same time points as for the blood chemistry examinations, and anti-rhG-CSFa antibody titers were determined using enzyme-linked immunosorbent assay (ELISA). The absorbance at 490 nm was read on a Bio-Rad Model 3550 Microplate Reader (Bio-Rad, USA).⁹

Statistical analysis

Statistical analyses were performed using Microsoft Office Excel 2007 and SPSS 17.0. The data were expressed as $\bar{x} \pm s$. Data on urinalyses, blood biochemistry, hematology analysis, and organ/body weight ratio were first analyzed for homogeneity of variance using Bartlett's test (Bartlett, 1937). In cases where homogeneity of variance was achieved, one-way analysis of variance (ANOVA) was applied. If the result was significant, Scheffe's test (Scheffe, 1959) was performed. When there was no homogeneity of variance by Bartlett's test, the data were analyzed by Kruskal-Wallis's H-test (Kruskal and Wallis, 1952).¹⁰ Data on body weight and food consumption were analyzed by repeated measures and multivariate ANOVA process of the general linear model and giving comparison among different groups and different measure time pairwise.¹¹ $P < 0.05$ represents statistical significance.

RESULTS

Results of routine examinations

During the course of the study, a male rat died after being treated with 10 $\mu\text{g/kg}$ rhG-CSFa for 14 days and three female rats in 10 $\mu\text{g/kg}$ dose group suffered from a partial alopecia in Day 52. These cases were judged to be incidental and unrelated to treatments. In addition, there were 4 male rats (4/10) with swelling on their joint of hind limbs in the highest dose group after treated with rhG-CSFa for 42 days. The symptom lasted for 7 days in two rats, and for the other two rats, lasted until the end of the study.

Animal growth curves for the 18-week study are illustrated in Figure 1. Mean body weight of the highest dose group was significantly lower than that of the control group in the early phase of the study ($P < 0.05$), but there was no difference after then. For food consumption, no test substance-related change was found.

Results of urinalyses

There was statistically significant difference in bilirubin between 500 $\mu\text{g/kg}$ dose group ($4.30 \pm 4.60 \mu\text{mol/L}$) and other groups (0) after treated with rhG-CSFa for 13 weeks, and differences in pH value among groups after 5-week recovery period (control group: 5.44 ± 0.56 , 1 $\mu\text{g/kg}$ dose group: 6.19 ± 0.75 , 10 $\mu\text{g/kg}$ dose group: 7.25 ± 0.65 , 100 $\mu\text{g/kg}$ dose group: 6.25 ± 0.71 , 500 $\mu\text{g/kg}$ dose group: 7.00 ± 0.84 ; all $P < 0.05$). No other significant differences in urinalyses were noted.

Blood biochemical results

Biochemistry values are represented in Table 1. After 13 weeks of treatment, mean BUN levels was the highest in male rats at 100 $\mu\text{g/kg}$ of rhG-CSFa ($P < 0.05$). The mean ALP levels increased significantly in male rats dosed at 10 and 500 $\mu\text{g/kg}$ of rhG-CSFa compared with control group ($P < 0.05$). The mean TP, ALB, ALP of the female rats in 10 and 100 $\mu\text{g/kg}$ dose groups and CHO of the 10 $\mu\text{g/kg}$ dose group were significantly higher than those in the control group (all $P < 0.05$). Following the 5-week recovery period, ALB, A/G levels had a statistically significant increase compared with the control group in male rats at the dose of 1 and 100 $\mu\text{g/kg}$ rhG-CSFa (all $P < 0.05$), and the same as TG levels at the dose of 1 and 10 $\mu\text{g/kg}$ rhG-CSFa. About female rats, TG levels of the 1 $\mu\text{g/kg}$ dose group and BUN, CPK levels in 500 $\mu\text{g/kg}$ dose group were significantly higher than those of the control group, and CHO level of the 10 $\mu\text{g/kg}$ dose group lower than that of the control group (all $P < 0.05$).

Hematological results

Hematological parameters are presented in Table 2. After 13 weeks of treatment, mean MPV levels significantly in-

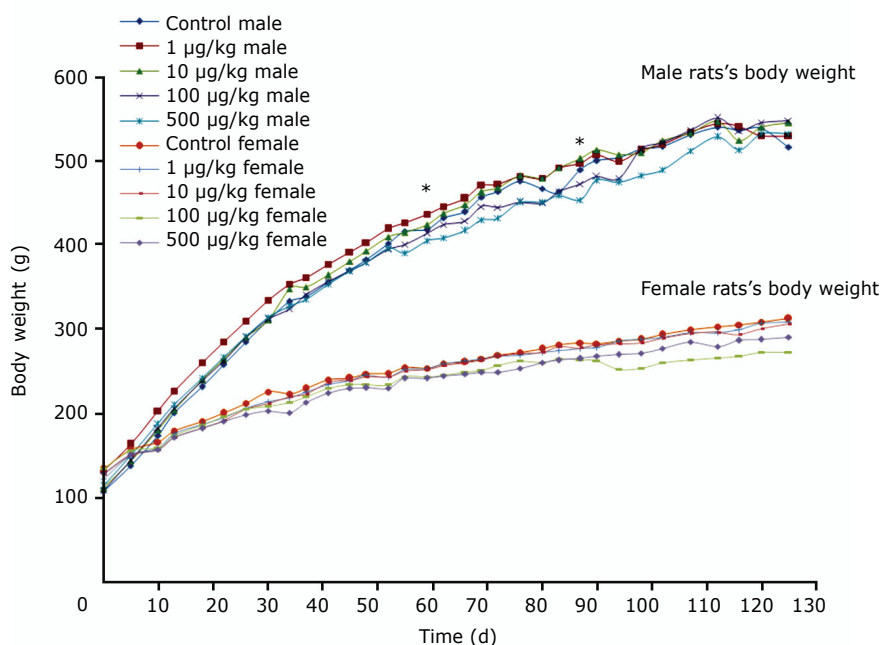


Figure 1. Body weight of rats treated subcutaneously with rhG-CSFa for 13 weeks and followed by 5-week-recovery period. rhG-CSFa: novel recombinant human granulocyte colony-stimulating factor.

* $P < 0.05$ the body weights of 500 µg/kg dose group were significantly lower than those of control group.

creased in male rats dosed at 500 µg/kg of rhG-CSFa, and decreased in female rats dosed at 100 µg/kg compared with control group (all $P < 0.05$). The mean PCT level was also lower than that of the control group in female rats dosed at 100 µg/kg ($P < 0.05$). In addition, the mean WBC count, neutrophils count, and neutrophils percentage were increased in male rats at the dose of 10 µg/kg or higher and lymphocytes percentage was decreased. Following the 5-week recovery period, the platelet and PCT levels had a statistically significant increase compared with the control group in male rats at the dose of 100 µg/kg, and the platelet levels did in female rats at the dose of 500 µg/kg (all $P < 0.05$). Other abnormalities did not follow dose-response and time-response relationship.

Results of gross pathology, organ weights, and histopathology

After 13 weeks of treatment, gross pathological examination showed enlargement of the spleen in 1 male rat in 1 µg/kg dose group and 2 male and 2 female rats in 500 µg/kg dose group, and the spleen/body weight ratio for the 500 µg/kg dose group (male: 0.61 ± 0.26 g/100 g, female: 0.33 ± 0.02 g/100 g) was higher than that for the control group (male: 0.14 ± 0.01 g/100 g, female: 0.31 ± 0.13 g/100 g; $P < 0.05$). Histopathological examination showed an increase in neutrophils in the red pulp of the spleen and leukocyte infiltration in the subcutis of injection site in a few

animals dosed at 100 µg/kg or higher dose group. Following the 5-week recovery period, all the abnormalities described as above were not detected. Other abnormalities, such as fatty degeneration of hepatocytes, lung focal lymphocyte accumulation, trachea chronic inflammation, chronic inflammation of joints wall, were observed in histopathological examination. Selected histopathological examinations are shown in Figure 2.

Anti-rhG-CSFa antibody

There was no anti-rhG-CSFa antibody detected in all the rats.

DISCUSSION

Compared with wild-type rhG-CSF, rhG-CSFa contains four additional amino acids at N terminal. This mutated molecule enhances the affinity of rhG-CSFa to bind to its target receptors and decreases the degradation rate in blood circulation system.⁶ As a result, it may increase the biological activity and prolong half life of rhG-CSFa. Both the previous pharmacological study and pharmacokinetic studies confirmed that rhG-CSFa may be a potentially more effective drug in clinic.^{5, 6} The obvious side effects of rhG-CSF in clinical use are joint pain, bone pain, muscle aches, and respiratory-like symptoms.

Table 1. Blood biochemical comparison in rats subcutaneously treated with rhG-CSFa for 13 weeks and followed by 5-week recovery period[§] (n=5)

Item			Control group		1 µg/kg		10 µg/kg		100 µg/kg		500 µg/kg	
					rhG-CSFa group		rhG-CSFa group		rhG-CSFa group		rhG-CSFa group	
13W	Male	TBIL(µmol/L)	0.90±	0.56	0.67±	0.35	0.57±	0.06	0.38±	0.17	0.38±	0.17
		TP(g/L)	63.33±	2.44	69.46±	8.74	68.53±	6.06	64.80±	2.46	63.72±	4.34
		ALB(g/L)	31.33±	0.75	31.78±	1.52	33.53±	2.40	30.83±	1.42	31.32±	1.67
		A/G	0.98±	0.06	0.87±	0.19	0.95±	0.05	0.91±	0.02	0.97±	0.07
		AST(U/L)	147.67±	18.77	225.20±	49.58	100.33±	29.02	148.00±	33.95	143.00±	36.51
		ALT(U/L)	43.33±	6.11	51.80±	24.51	34.33±	5.77	38.00±	14.18	37.75±	2.87
		ALP(U/L)	82.60±	22.02	74.00±	13.45	177.00±	17.56*	430.00±	101.08	956.00±	159.82*
		GLU(mmol/L)	6.75±	0.37	5.37±	0.10	7.67±	3.61	6.95±	1.59	6.92±	0.62
		BUN(mmol/L)	6.77±	1.33	6.54±	0.28	5.55±	1.19	8.74±	1.49*	7.66±	2.14
		CRE(µmol/L)	28.10±	5.83	32.90±	2.50	29.00±	1.04	36.33±	4.37	31.03±	4.03
		TG(mmol/L)	0.76±	0.17	0.75±	0.09	1.10±	0.42	0.58±	0.13	0.83±	0.13
		CHO(mmol/L)	1.42±	0.34	1.44±	0.25	1.28±	0.12	1.47±	0.22	1.24±	0.16
	CPK(U/L)	673.00±	182.35	523.20±	137.50	566.67±	128.08	564.33±	145.57	553.50±	183.19	
	Female	TBIL(µmol/L)	1.10±	0.52	1.05±	0.44	1.05±	0.28	1.04±	0.32	1.07±	0.25
		TP(g/L)	66.82±	3.86	66.15±	1.81	76.12±	6.03*	73.92±	2.45*	65.96±	3.07
		ALB(g/L)	33.00±	2.76	34.25±	1.41	37.55±	4.27*	37.52±	1.80*	31.48±	3.35
		A/G	0.98±	0.10	1.08±	0.15	0.97±	0.07	1.06±	0.09	0.87±	0.12
		AST(U/L)	123.00±	19.61	102.25±	19.80	103.00±	19.41	108.60±	23.16	144.50±	16.77
		ALT(U/L)	35.50±	7.19	44.25±	5.88	30.25±	9.25	31.80±	4.76	35.25±	6.52
		ALP(U/L)	54.75±	8.22	56.75±	9.14	81.00±	7.07*	101.50±	14.62*	286.00±	209.00
		GLU(mmol/L)	4.87±	0.62	5.59±	0.41	6.03±	0.81	6.23±	0.58	7.05±	0.94
		BUN(mmol/L)	7.68±	1.14	7.61±	0.89	7.44±	1.47	6.38±	0.61	7.33±	0.89
		CRE(µmol/L)	35.40±	4.33	42.30±	6.12	32.85±	2.46	34.54±	1.89	38.95±	3.09
		TG(mmol/L)	0.65±	0.09	0.71±	0.19	0.82±	0.12	0.84±	0.33	0.67±	0.07
CHO(mmol/L)		1.52±	0.11	1.40±	0.60	2.14±	0.50*	2.06±	0.12	1.68±	0.30	
CPK(U/L)	431.25±	48.36	465.00±	49.64	456.75±	47.99	474.20±	41.81	437.50±	39.48		
5W	Male	TBIL(µmol/L)	0.48±	0.18	0.47±	0.16	0.45±	0.12	0.45±	0.15	0.47±	0.12
		TP(g/L)	65.16±	3.83	62.12±	5.56	62.58±	1.84	67.65±	5.59	65.48±	2.18
		ALB(g/L)	31.58±	1.89	34.95±	3.37*	30.78±	2.67	35.78±	2.25*	32.95±	1.08
		A/G	0.94±	0.04	1.28±	0.05*	0.98±	0.19	1.12±	0.05*	1.02±	0.05
		AST(U/L)	170.6±	26.14	137.5±	17.62*	106.25±	19.89*	145.00±	9.20*	130.00±	31.84
		ALT(U/L)	49.20±	7.54	49.50±	3.40	59.00±	5.64	47.75±	6.18	47.00±	3.92
		ALP(U/L)	65.00±	23.36	83.00±	25.81	151.50±	47.95*	63.50±	7.85*	87.75±	26.15
		GLU(mmol/L)	4.66±	0.47	8.81±	1.33*	8.96±	0.62*	7.60±	1.14*	6.62±	1.13*
		BUN(mmol/L)	6.84±	0.52	6.26±	0.78	6.48±	0.31	6.55±	0.88	6.75±	0.82
		CRE(µmol/L)	40.66±	3.26	50.10±	4.39*	37.68±	4.12	41.98±	5.51	39.10±	4.20
		TG(mmol/L)	0.65±	0.19	1.15±	0.55*	1.16±	0.44*	0.70±	0.18	0.74±	0.18
		CHO(mmol/L)	1.20±	0.16	1.12±	0.15	1.01±	0.22	1.04±	0.24	1.14±	0.29
	CPK(U/L)	649.40±	24.51	598.00±	29.84	579.00±	28.57	620.50±	29.37	592.75±	23.36	
	Female	TBIL(µmol/L)	1.00±	0.22	0.97±	0.33	1.25±	0.35	0.95±	0.35	0.96±	0.34
		TP(g/L)	72.80±	6.72	70.80±	7.20	72.07±	10.76	70.70±	2.87	71.72±	5.91
		ALB(g/L)	40.15±	3.42	37.75±	2.04	36.52±	1.90	39.85±	3.44	37.76±	2.71
		A/G	1.23±	0.04	1.15±	0.15	1.20±	0.05	1.30±	0.17	1.12±	0.08
		AST(U/L)	84.50±	5.86	84.50±	7.72	99.75±	6.32	94.00±	5.45	107.60±	5.37
		ALT(U/L)	26.25±	4.20	27.50±	4.12	31.50±	4.94	28.50±	4.65	27.00±	5.22
		ALP(U/L)	40.00±	6.68	39.00±	8.12	45.00±	4.30	39.50±	7.60	38.40±	6.49
		GLU(mmol/L)	6.62±	1.12	6.54±	0.79	6.32±	0.66	6.23±	1.12	6.12±	0.68
		BUN(mmol/L)	5.72±	0.61	7.20±	1.48	5.50±	0.98	7.25±	0.34	8.12±	0.70*
		CRE(µmol/L)	42.55±	2.16	41.17±	3.86	38.75±	2.87	45.07±	4.22	40.38±	3.65
		TG(mmol/L)	0.80±	0.39	1.38±	0.54*	1.13±	0.16	0.96±	0.29	0.92±	0.41
CHO(mmol/L)		1.34±	0.28	1.12±	0.08	1.12±	0.10*	1.30±	0.09	1.31±	0.15	
CPK(U/L)	181.75±	35.58	252.25±	36.68	257.00±	120.58	216.00±	74.96	456.80±	84.66*		

§: Plus-minus values are means±SD.

13W: rats sacrificed after treated with rhG-CSFa for 13 weeks; 5W: rats sacrificed after 5-week recovery period; TBIL: total bilirubin; TP: total protein; ALB: serum albumin; A/G: albumin/globulin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GLU: glucose; BUN: urea nitrogen; CRE: creatinine; TG: triglyceride; CHO: total cholesterol; CPK: phosphokinase.

*P<0.05 compared with control group.

Table 2. Hematological values in rats subcutaneously treated with rhG-CSFa for 13 weeks and followed by 5-week recovery period^S (n=5)

Item		Control group	1 µg/kg		10 µg/kg		100 µg/kg		500 µg/kg	
			rhG-CSFa group		rhG-CSFa group		rhG-CSFa group		rhG-CSFa group	
13W	Male	WBC(×10 ⁹ /L)	9.87± 1.53	6.42± 1.74	13.40± 4.89*	32.23± 13.34*	98.75± 17.22*			
		NE(×10 ⁹ /L)	0.93± 0.28	1.78± 0.25	5.50± 0.69*	18.97± 5.54*	75.87± 12.67*			
		LY(×10 ⁹ /L)	8.33± 1.36	4.55± 1.73	6.60± 2.32	12.17± 6.59	21.00± 8.90*			
		MO(×10 ⁹ /L)	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.30± 0.10	1.72± 0.91			
		EO(×10 ⁹ /L)	0.07± 0.04	0.10± 0.00*	0.00± 0.00	0.00± 0.00	0.15± 0.17*			
		BA(×10 ⁹ /L)	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00			
		NE%(%)	9.57± 2.60	22.58± 12.76	36.72± 21.93*	59.47± 20.86*	74.30± 14.17*			
		LY%(%)	89.53± 3.00	75.22± 11.90	62.70± 11.60*	36.80± 16.14*	23.45± 13.16*			
		MO%(%)	0.27± 0.29	0.25± 0.26	0.13± 0.05	3.60± 4.95*	2.02± 1.18*			
		EO%(%)	0.63± 0.15	1.95± 1.33	0.45± 0.38	0.13± 0.06	0.22± 0.22			
		BA%(%)	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00			
		RBC(×10 ¹² /L)	8.23± 0.25	8.30± 0.58	8.52± 0.86	8.24± 0.35	7.53± 0.82			
		HGB(g/L)	158.33± 10.41	159.50± 13.77	163.25± 13.96	165.67± 12.90	188.00± 10.10*			
		HCT	39.83± 0.76	39.55± 3.87	42.12± 4.26	41.10± 2.96	41.15± 2.49			
		MCV(fL)	48.40± 2.36	47.62± 1.94	49.23± 0.90	49.87± 2.22	54.80± 2.65			
		MCH(pg)	19.23± 1.53	19.22± 0.72	19.20± 0.64	20.10± 0.92	25.25± 3.91			
		MCHC(g/L)	397.33± 22.50	403.75± 5.68	388.25± 17.11	403.00± 5.29	442.33± 46.49			
		RDW(%)	12.07± 0.38	11.98± 0.22	12.02± 0.39	12.10± 0.10	12.92± 1.11			
		PLT(×10 ⁹ /L)	718.67± 43.68	442.50± 209.97	726.50± 115.29	516.67± 151.78	574.00± 159.43			
		PCT	0.12± 0.01	0.09± 0.02	0.15± 0.04	0.11± 0.02	0.08± 0.03			
		MPV(fL)	1.67± 0.21	2.30± 0.71	2.05± 0.24	2.10± 0.26	2.65± 0.33*			
		PDW(fL)	14.23± 0.58	15.85± 0.72	14.80± 0.20	16.23± 1.10	17.10± 1.19			
		PT(s)	19.70± 1.57	18.55± 3.18	22.19± 2.10	19.45± 0.21	24.05± 4.76			
	Female	WBC(×10 ⁹ /L)	8.10± 2.42	7.32± 2.28	6.42± 1.21	5.12± 0.86	20.68± 9.87*			
		NE(×10 ⁹ /L)	1.10± 0.92	1.97± 1.63	3.32± 1.21*	0.92± 0.11	13.87± 10.63*			
		LY(×10 ⁹ /L)	6.95± 1.50	5.27± 2.78	3.10± 0.18	4.18± 0.94	6.57± 0.99			
		MO(×10 ⁹ /L)	0.00± 0.00	0.02± 0.05	0.00± 0.00	0.00± 0.00	0.17± 0.21			
		EO(×10 ⁹ /L)	0.05± 0.05	0.00± 0.00	0.00± 0.00	0.02± 0.04	0.05± 0.06			
		BA(×10 ⁹ /L)	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00			
		NE%(%)	11.97± 6.96	27.47± 22.13	50.20± 9.15	18.76± 4.61	58.82± 23.28			
		LY%(%)	87.17± 7.11	71.22± 22.65	48.95± 9.04	79.92± 4.20	40.07± 23.55			
		MO%(%)	0.10± 0.08	0.45± 0.21	0.42± 0.22	0.54± 0.25	0.72± 0.51			
		EO%(%)	0.70± 0.14	0.85± 1.23	0.40± 0.08	0.70± 0.47	0.35± 0.26			
		BA%(%)	0.05± 0.06	0.00± 0.00	0.02± 0.05	0.04± 0.05	0.02± 0.05			
		RBC(×10 ¹² /L)	8.00± 0.31	7.72± 0.31	7.73± 1.03	7.87± 0.52	7.38± 0.11			
		HGB(g/L)	155.75± 9.74	146.75± 9.84	151.50± 21.58	154.60± 11.41	157.25± 4.11			
		HCT	39.22± 2.93	38.05± 2.15	38.60± 5.32	40.66± 3.27	38.77± 1.18			
		MCV(fL)	49.07± 2.87	49.25± 1.25	49.90± 0.83	51.66± 1.09	52.50± 1.14			
		MCH(pg)	19.50± 1.15	19.00± 0.95	19.58± 0.22	19.66± 0.44	21.33± 0.56			
		MCHC(g/L)	397.50± 9.88	385.50± 10.88	392.25± 8.96	380.40± 10.45	405.50± 7.19			
		RDW(%)	11.55± 0.41	11.77± 0.39	11.77± 0.32	11.74± 0.23	11.80± 0.73			
		PLT(×10 ⁹ /L)	761.75± 37.78	697.75± 80.60	704.50± 68.33	690.40± 123.93	826.25± 72.94			
		PCT	0.18± 0.01	0.14± 0.03	0.14± 0.01	0.13± 0.02*	0.15± 0.06			
		MPV(fL)	2.38± 0.15	2.00± 0.32	1.92± 0.28	1.86± 0.35*	2.20± 0.49			
		PDW(fL)	14.70± 0.47	15.07± 1.11	14.82± 0.22	15.06± 0.83	14.67± 0.21			
		PT(s)	22.30± 1.71	17.55± 2.11	22.10± 2.38	17.85± 2.96	20.23± 3.35			
5W	Male	WBC(×10 ⁹ /L)	7.46± 1.19	8.65± 2.71	6.57± 0.31	6.87± 0.71	8.27± 0.69			
		NE(×10 ⁹ /L)	0.52± 0.15	0.32± 0.26	0.60± 0.61	0.45± 0.13	0.60± 0.36			
		LY(×10 ⁹ /L)	6.94± 1.10	8.27± 2.44	5.85± 2.05	6.40± 0.67	7.65± 1.17			
		MO(×10 ⁹ /L)	0.00± 0.00	0.05± 0.05	0.02± 0.05	0.02± 0.05	0.02± 0.05			
		EO(×10 ⁹ /L)	0.00± 0.00	0.05± 0.06	0.03± 0.05*	0.03± 0.05	0.03± 0.05			
		BA(×10 ⁹ /L)	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00			
		NE%(%)	6.92± 1.32	3.72± 1.64	4.57± 0.60	6.25± 1.93	7.37± 4.77			
		LY%(%)	92.62± 1.32	96.62± 1.59	89.82± 9.56	92.87± 1.61	92.15± 4.73			
		MO%(%)	0.12± 0.08	0.05± 0.06	0.22± 0.25	0.20± 0.14	0.10± 0.08			
		EO%(%)	0.34± 0.11	0.57± 0.22	0.80± 0.26	0.62± 0.46	0.37± 0.10			

(Continued)

Item	Control group		1 µg/kg rhG-CSFa group		10 µg/kg rhG-CSFa group		100 µg/kg rhG-CSFa group		500 µg/kg rhG-CSFa group	
BA%(%)	0.00±	0.00	0.02±	0.05	0.02±	0.05	0.05±	0.06	0.00±	0.00
RBC($\times 10^9/L$)	8.86±	0.38	8.83±	0.51	9.02±	0.44	9.19±	0.51	8.29±	0.34
HGB(g/L)	165.00±	5.48	153.00±	6.05	153.25±	8.65	164.75±	11.81	152.00±	5.48
HCT	43.02±	1.46	42.52±	1.06	42.97±	2.83	45.40±	2.66	40.90±	0.87
MCV(fL)	48.60±	1.37	48.20±	1.85	47.65±	2.27	49.40±	2.19	49.37±	1.79
MCH(pg)	18.64±	0.48	17.37±	0.92	17.00±	1.04	17.92±	1.10	18.35±	0.87
MCHC(g/L)	383.60±	4.22	359.50±	8.66	357.00±	10.23	362.75±	5.91	371.75±	10.59
RDW(%)	11.54±	0.27	12.10±	0.36*	12.35±	0.52*	11.75±	0.42	11.85±	0.31
PLT($\times 10^9/L$)	717.80±	102.94	663.75±	137.23	703.25±	74.73	870.00±	56.08*	605.75±	128.81
PCT	0.14±	0.05	0.15±	0.03	0.14±	0.02	0.20±	0.01*	0.12±	0.04
MPV(fL)	1.92±	0.50	2.35±	0.31	1.97±	0.21	2.32±	0.10	1.87±	0.36
PDW(fL)	14.62±	0.56	15.05±	0.54	14.97±	0.54	15.10±	0.73	15.22±	0.63
PT(s)	20.84±	0.85	21.27±	1.57	21.00±	5.30	21.95±	1.26	21.85±	0.23
Female WBC($\times 10^9/L$)	4.34±	1.07	4.45±	0.97	4.66±	2.74	3.57±	0.40	5.38±	1.18
NE($\times 10^9/L$)	0.44±	0.31	0.20±	0.11	0.28±	0.22	0.13±	0.06	0.54±	0.29
LY($\times 10^9/L$)	3.9 ±	0.89	4.22±	0.88	5.48±	1.48*	3.43±	0.35	4.84±	0.94
MO($\times 10^9/L$)	0.00±	0.00	0.00±	0.00	0.00±	0.00	0.00±	0.00	0.00±	0.00
EO($\times 10^9/L$)	0.00±	0.00	0.03±	0.05	0.10±	0.00*	0.00±	0.00	0.00±	0.00
BA($\times 10^9/L$)	0.00±	0.00	0.00±	0.00	0.00±	0.00	0.00±	0.00	0.00±	0.00
NE%(%)	9.94±	6.84	3.95±	2.41	4.54±	1.81	4.27±	1.53	9.62±	3.08
LY%(%)	89.14±	7.01	95.10±	2.51	93.98±	1.49	95.10±	1.51	89.84±	2.98
MO%(%)	0.28±	0.36	0.22±	0.22	0.16±	0.11	0.07±	0.06	0.16±	0.09
EO%(%)	0.60±	0.28	0.72±	0.38	1.28±	0.41*	0.57±	0.30	0.34±	0.15
BA%(%)	0.04±	0.05	0.00±	0.00	0.04±	0.05	0.00±	0.00	0.04±	0.09
RBC($\times 10^9/L$)	7.63±	0.56	7.33±	0.21	7.38±	0.10	7.27±	0.42	8.30±	0.83
HGB(g/L)	152.80±	10.85	145.25±	6.95	145.20±	4.49	138.67±	5.03	159.20±	13.29
HCT	39.18±	2.84	37.65±	0.69	37.76±	1.00	36.23±	0.70	41.88±	5.10
MCV(fL)	51.34±	1.15	51.37±	2.01	51.16±	1.46	49.97±	3.29	50.46±	2.36
MCH(pg)	19.92±	0.48	19.82±	1.34	19.66±	0.72	19.17±	1.55	18.44±	1.84
MCHC(g/L)	389.60±	10.43	385.75±	16.98	384.60±	7.23	382.33±	6.81	381.40±	17.23
RDW(%)	11.34±	0.24	11.17±	0.22	11.40±	0.34	11.17±	0.30	11.34±	0.40
PLT($\times 10^9/L$)	657.80±	115.25	671.25±	36.26	493.00±	52.88*	686.67±	145.72	810.60±	150.74*
PCT	0.13±	0.03	0.12±	0.01	0.07±	0.01	0.12±	0.03	0.17±	0.04
MPV(fL)	2.02±	0.40	1.87±	0.19	1.44±	0.21	1.67±	0.15	2.02±	0.28
PDW(fL)	15.16±	0.78	15.17±	0.29	15.88±	0.46	15.33±	0.38	14.84±	0.21
PT(s)	14.28±	2.29	18.68±	0.68	19.26±	1.50	16.93±	6.57	17.70±	1.59*

§: Plus-minus values are means±SD.

WBC: white blood cell count; NE: neutrophils count; LY: lymphocytes count; MO: monocyte count; EO: eosinophils count; BA: basophils count; NE%: neutrophils percentage; LY%: lymphocytes percentage; MO%: monocyte percentage; EO%: eosinophils percentage; BA%: basophils percentage; RBC: red blood cell count; HGB: hemoglobin concentration; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean cell hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red blood cell distribution width; PLT: platelet count; PCT: plateletcrit; MPV: mean platelet volume; PDW: platelet distribution width; PT: prothrombin time.

* $P < 0.05$ compared with control group.

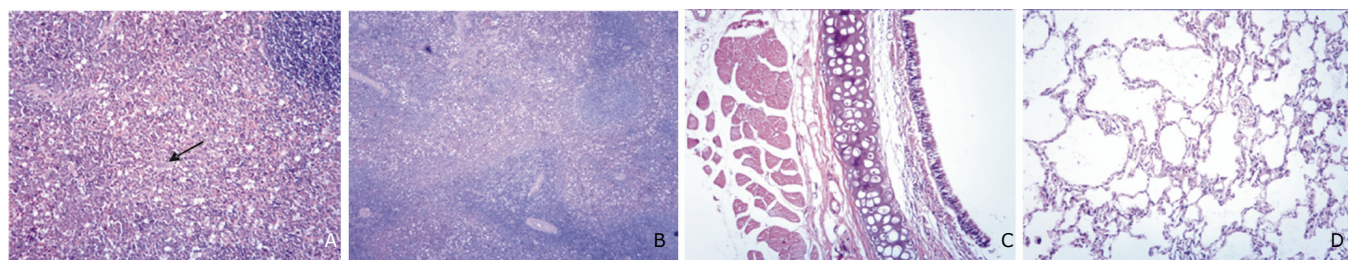


Figure 2. Histopathological results of selected tissues stained with hematoxylin and eosin. $\times 10$

A. Macrophage proliferation in spleen (arrow) of rats after treated with 500 µg/kg of rhG-CSFa for 13 weeks.

B. Spleen tissue has no abnormality after 5-week-recovery period in 500 µg/kg dose group.

C. Trachea tissue has no abnormality after 5-week-recovery period in 500 µg/kg dose group.

D. Lung tissue has no abnormality after 5-week-recovery period in control group.

In our studies, no rhG-CSFa-related abnormalities were observed in body weight and food consumption measurement in any study group. Although there were statistically significant differences in their bilirubin and pH values in urinalyses, these differences were not considered toxicologically meaningful. Although there was significant difference about several parameters in blood biochemical examinations between groups, these effects were sporadic, random phenomena with no dose-response and time-response relationship, so we concluded that all of these had no biological significance. The hematological analyses revealed that the mean WBC count, neutrophils count, and neutrophils percentage were increased in male rats at the dose of 10 µg/kg or higher, and these indicated that the rhG-CSFa could enhance granulocytes proliferation. The difference in platelet count as well as PCT suggested rhG-CSFa may increase the number of platelet, but more conclusive studies are warranted. Other abnormalities did not follow dose-response and time-response relationship, so we concluded these small scale fluctuations had no biological significance. Some abnormalities were observed in the spleen of a few rats treated with highest dose of rhG-CSFa, but these abnormalities were recovered within 5-week recovery period, suggesting that the toxicity is reversible. And all abnormalities in histopathology appeared to be randomly distributed among treated and control groups and could occur as spontaneous disease in rats, so we attributed them to normal background biological variability. No significant immunogenicity was observed with rhG-CSFa administration to rats in the chronic toxicity studies.

In conclusion, the molecular addition and site mutation of rhG-CSFa did not produce any toxicological effect and rhG-CSFa had no significant toxicity and immunogenicity.

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