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Original Article

Effect and mechanism of recombinant human granulocyte colony-stimulating factor on diarrhea caused by leukopenia in New Zealand rabbits

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Abstract: Objective: The purpose of this paper was to explore the mechanism and effect of recombinant human granulocyte colony stimulating factor (rhG-CSF) on diarrhea triggered by leukopenia in New Zealand rabbits. Methods: Forty rabbits were injected with the cyclophosphamide (CTX, 50 mg/kg) through ear vein for 4 consecutive days to lead to the leukopenia. The leukopenic rabbits were randomly divided into two groups (n=20/group): rhG-CSF group (injected with rhG-CSF 5 μg/kg for 7 consecutive days) and control group (injected with normal saline). The general condition was monitored daily. The blood routine was evaluated by automatic blood cell count analyzer and the immunoglobulin A (IgA), IgG, and IgM levels were assessed by the enzyme-linked immunosorbent assay (ELISA) before administering the first dose of CTX, and on days 0, 2, 5, 7, 11, and 14 after the last dose. The bone marrow was extracted using medullo-puncture needle, smeared, stained by hematoxylin-eosin and examined before the first dose of CTX, and on days 2 and 7 after the last dose. *Results:* Compared to the control group, the rates of diarrhea and mortality of model rabbits in the rhG-CSF group were decreased ($P<0.05$); the peripheral white blood cell (WBC) count and the IgA, IgG, and IgM were increased ($P<0.05$); the bone marrow proliferation was active. *Conclusion:* rhG-CSF could reduce the occurrence rate of diarrhea and mortality due to leucopenia by improving the number of WBC in peripheral blood and the expression efficiency of IgG, IgA, and IgM in rabbits.

Keywords: Recombinant human granulocyte colony stimulating factor, leukopenia, diarrhea, mortality, immunoglobulin

Introduction

In 2012, about 14 million new cases of malignant tumors were detected worldwide, and mortality from malignant tumors reached 8.2 million [1]. It causes a great social and economic burden to the society. Chemotherapy is widely used in treatment for several types of solid tumor [2]. However, several chemotherapeutic agents, such as busulfan, nitrosourea, and mitomycin C are reported to induce myelosuppression commonly during tumor chemotherapy [3, 4]. The inhibition of granulocyte-macrophage cell lines which is one presentation of the myelosuppression can result in leukopenia [5-7]. In addition, the severe secondary infections can be triggered by leukopenia and it leads to a fail of chemotherapy in clinic [7]. Recombinant human granulocyte colony stimulating factor (rhG-CSF) can promote granulocyte proliferation, differentiation, and functional activation as well as mobilization of peripheral blood progenitor cells, and thereby increase white blood count (WBC) and treat chemotherapy-induced leukopenia [8, 9]. Moreover, it can relieve or prevent the bone marrow suppression caused by chemotherapy, promote the recovery of bone marrow hematopoietic function, reduce the symptoms of infection and consequent diarrhea without any obvious side ef-

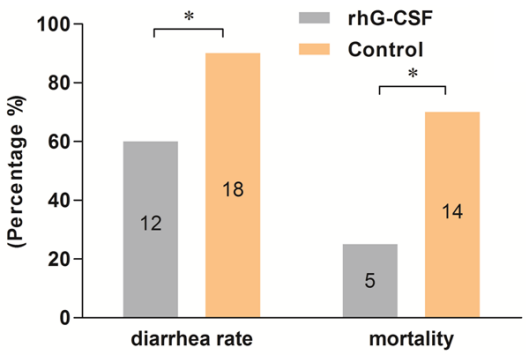

Figure 1. Comparison of the rates of diarrhea and mortality between rhG-CSF group and control group in New Zealand rabbits, *indicates $P<0.05$, which is statistically significant. Diarrhea rate: $\chi^2=4.800$, $P<0.05$; mortality: $\chi^2=8.120$, $P<0.05$.

fects. Therefore, rhG-CSF has become the key factor to ensure the successful completion [10] and improve the efficacy of chemotherapy in clinic [11]. Recently, rhG-CSF is widely used in the treatment of leukopenia caused by chemotherapy and bone marrow transplantation [12].

The rhG-CSF is used to treat myelosuppression after chemotherapy, which can improve the recovery of bone marrow suppression evaluated by a routine blood changes in clinical practice and reduce the incidence of secondary infection [13, 14]. However, the underlying mechanism has not yet been elucidated. In our study, the rabbits' leukopenia model was established by injecting cyclophosphamide (CTX), and a treatment of rhG-CSF was carried out to reveal the mechanism. The mechanism uncovered will provide experimental support and direction for the rhG-CSF treatment on myelosuppression and the secondary diseases, such as diarrhea.

Materials and methods

Establishment of the animal leucopenia model

The experiment was approved by the Ethics Committee of Chongqing Medical University. A total of 40 male and female New Zealand rabbits, weighing 2.0-2.5 kg, were provided by the Animal Experimental Center of Chongqing Medical University [production license: SCXK (Chongqing) 2012-0001]. These animals were maintained on a standard 12 h light -12 h dark cycle, in a temperature-controlled environment (23±2°C), with free access to water and food. 50 mg/kg of CTX (Shanxi Pude Pharmaceutical Co., Ltd., China) was injected daily for 4 consecutive days through ear vein. Rabbits were randomly divided into rhG-CSF group and control group (n=20/group) after the last dose of CTX. Then, the 5 μg/kg rhG-CSF (Beijing Shuanglu Pharmaceutical Co., Ltd., China) was injected into the ear vein daily for 7 consecutive days in the rhG-CSF group. The control group was treated with normal saline instead of rhG-CSF.

General condition

To investigate the effect of rhG-CSF on the general condition of the animals, eating, drinking, excretion, mental status, diarrhea, and mortality of the rabbits were observed daily. The details of excretion such as times of defecation, fecal volume, fecal matter, and the duration of diarrhea were observed and recorded carefully.

Blood cell count

To study the influence of rhG-CSF on blood routine, 1 mL blood was withdrawn to the anticoagulant tube containing EDTA (Health medical products Co., Ltd., Jiangsu China) from the ear artery of rabbits before administering the first dose of CTX and on days 0, 2, 5, 7, 11, and 14 after the last dose. The routine blood test was detected by an automatic blood cell count analyzer (Bowlinman Sunshine Co., Ltd, Beijing, China).

Ig expression efficiency

To evaluate the effect of rhG-CSF on Ig, 0.5 mL blood was withdrawn from the ear artery of each rabbit before administering the first dose of CTX and on days 0, 2, 5, 7, 11, and 14 after the final dose. The serum was isolated by centrifugation at 3000 rpm for 15 min. The IgA, IgG, and IgM were detected using the ELISA kits (Hu Shang Biotechnology Co., Ltd. Shanghai, China) according to the manufacturer's instructions. The upper and lower limits of the normal range of IgA, IgG, and IgM in serum were calculated by 95% confidence interval (CI).

Bone marrow changes

To assess the effect of rhG-CSF on bone marrow, the bone marrow samples were extracted using medullo-puncture needle before the first administration of CTX and on days 2 and 7 after the final administration and then fixed in 4% paraformaldehyde (Chongqing Boer Biotechno-

Table 1. The upper and lower limits of normal values for peripheral blood cells and Ig in New Zealand rabbits

Item	WBC $\times 10^9$/L	Neutrophil $\times 10^9$/L	Lymphocyte $\times 10^9$/L	Monocyte $\times 10^9$/L	IgA (μg/mL)	IgG (mg/mL)	IgM (μg/mL)
Upper limit	12.19	5.99	6.55	0.64	93.36	1.28	84.73
Lower limit	8.78	3.37	3.81	0.51	73.19	0.88	62.86

Table 2. Changes in white blood cell count after rhG-CSF treatment ($\bar{x} \pm s$)

Group	n	0 d	2 d	5 d	7 d	11 d	14 d
rhG-CSF	20	2.29±1.11	15.06±7.41	16.32±6.70	7.38±1.84	7.78±3.34	6.07±0.43
Control	20	2.18±1.03	8.13±1.30	7.34±1.19	6.43±0.67	4.14±0.34	4.76±0.86
Repeated measures analysis of variance							
Tests of Within-Subject Effects		Tests of Between-Subject Effects (P=0.017)					
t (P=0.000)	Time*group (P=0.143)						
t-test	rhG-CSF vs. control	P=0.904	P=0.178	P=0.072	P=0.444	P=0.126	P=0.045

logy Co., Ltd., China). The samples were dehydrated, embedded, sliced, stained with hematoxylin-eosin, and observed under a microscope (Olympus Co., Japan).

Statistical analysis

All the data were expressed as mean ± SD. SPSS 22.0 software (IBM, US) was used for statistical analysis. Repeated measure analysis of variance and *t*-test were used to analyze the difference of the blood cell count and immunoglobulin level in groups. Chi-square test was used to analyze the differences in the rates of diarrhea and mortality. The *P*-value less than 0.05 was considered statistically significant. The *P*-value less than 0.01 was regarded as highly statistically significant.

Result

Diarrhea rate and mortality

The rabbits showed adverse reactions after CTX injection, including a decrease in the intake of food and water, reduction of activities and lying huddled up in the cage without response, and appearance of diarrhea. The diarrhea was relieved rapidly after using rhG-CSF and any new cases were not observed on the 4th day after rhG-CSF injection. However, the diarrhea reappeared after using rhG-CSF for 7 days, followed by mortality. During the observation period, 12 cases of diarrhea and 5 deaths were noted in the rhG-CSF group; the rate of diarrhea was 60% and mortality was 25%. Moreover, the diarrhea of the control group was also not relieved, 18 cases of diarrhea (90%) and 14 deaths with the mortality (70%) were observed. The rates of diarrhea and mortality were statistically significant in the rhG-CSF group as compared to the control group ($P<0.05$) (**Figure 1**). The results showed that rhG-CSF can reduce the rate of occurrence of diarrhea and mortality in rabbit.

WBC count in blood routine

To understand the reason of rhG-CSF can reduce the rates of diarrhea and mortality, routine blood tests were applied. No abnormal cells were found in all blood samples. The upper and lower limits of the normal values for peripheral blood cells were as follows (**Table 1**).

The difference in the WBC count measured at different time points was statistically significant ($P<0.05$) and was not affected by time and the different treatment methods between two groups ($P>0.05$). The difference in the WBC between the rhG-CSF and control groups was statistically significant ($P<0.05$). The WBC count in the rhG-CSF group was significantly higher than that of the control group on the 14th day ($P<0.05$) (**Table 2**).

The counts of neutrophil, lymphocyte and monocyte-major parts in WBC- were measured respectively to observe the change of WBC:

● The difference in the neutrophil count measured at different time was not statistically significant ($P>0.05$) and was not affected by time and the different treatment methods between

Table 3. Changes in neutrophil count after rhG-CSF treatment ($\bar{x}\pm s$)

Group	n	0 d	2 d	5 d	7 d	11 d	14 d
rhG-CSF	20	0.28±0.21	7.21±5.59	11.27±7.28	3.56±0.88	4.63±3.44	3.09±0.60
Control	20	0.20±0.18	4.57±1.12	3.86±1.00	3.13±0.65	1.95±0.38	2.01±0.31
Repeated measures analysis of variance							
Tests of Within-Subject Effects		Tests of Between-Subject Effects (P=0.025)					
t (P=0.080)	Time*group (P=0.340)						
t-test	rhG-CSF vs. control	P=0.619	P=0.466	P=0.134	P=0.520	P=0.247	P=0.039

Table 4. Changes in lymphocyte count after rhG-CSF treatment ($\bar{x}\pm s$)

Group	n	0 d	2 d	5 d	7 d	11 d	14 d
rhG-CSF	20	1.65±0.81	5.95±1.86	3.71±0.86	2.93±0.96	2.20±0.69	2.35±0.22
Control	20	1.58±0.54	2.55±0.17	2.51±0.53	2.60±0.62	1.85±0.31	2.24±0.48
Repeated measures analysis of variance							
Tests of Within-Subject Effects		Tests of Between-Subject Effects (P=0.031)					
t (P=0.000)	Time*group (P=0.006)						
t-test	rhG-CSF vs. control	P=0.902	P=0.034	P=0.090	P=0.630	P=0.454	P=0.704

Table 5. Changes in monocyte count after rhG-CSF treatment ($\bar{x}\pm s$)

Group	n	0 d	2 d	5 d	7 d	11 d	14 d
rhG-CSF	20	0.04±0.01	1.15.±0.59	0.44±0.19	0.35±0.13	0.62±0.71	0.24±0.21
Control	20	0.04±0.03	0.51±0.46	0.29±0.08	0.33±0.30	0.19±0.02	0.22±0.11
Repeated measures analysis of variance							
Tests of Within-Subject Effects		Tests of Between-Subject Effects (P=0.135)					
t (P=0.048)	Time*group (P=0.389)						
t-test	rhG-CSF vs. control	P=0.872	P=0.185	P=0.277	P=0.946	P=0.350	P=0.907

two groups ($P>0.05$). The difference in the neutrophil count between the two group was statistically significant ($P<0.05$). The neutrophil count was significantly higher in the rhG-CSF group as compared to the control group on the 14th day ($P<0.05$) (**Table 3**).

● The difference in the lymphocyte count measured at different time was statistically significant ($P<0.05$) and affected by time and the different treatment methods between two groups ($P<0.05$). The difference in the lymphocyte count between the rhG-CSF and control groups was statistically significant ($P<0.05$). The lymphocyte count in the rhG-CSF group was significantly higher than that in the control group on the 2nd day ($P<0.05$) (**Table 4**).

● The difference in the monocyte count measured at different time points was statistically significant ($P<0.05$) and not affected by time and the different treatment methods between two groups ($P>0.05$). The difference in the monocyte count between the rhG-CSF and control groups was not statistically significant ($P>0.05$, **Table 5**).

The difference in hemoglobin and platelet count between the rhG-CSF and control groups was not statistically significant ($P>0.05$).

All above results showed that rhG-CSF could increase the number of leukocytes, thereby increasing the anti-infection ability of rabbits in the rhG-CSF group.

Ig expression efficiency

To explore more anti-infection mechanism of rhG-CSF, the expression efficiency of Ig was detected.

The difference in the IgA expression level measured at different time was statistically significant ($P<0.05$) in a time-dependent manner. It was also influenced by the different treatment

Table 6. Changes in IgA count after rhG-CSF treatment ($\bar{x}\pm s$)

Group	n	0 d	2 d	5 d	7 d	11 d	14 d
rhG-CSF	20	75.62±48.00	69.43±26.95	94.40±6.47	120.22±38.75	186.70±6.08	173.68±5.59
Control	20	75.87±28.95	80.03±10.27	62.53±18.78	61.36±14.26	37.35±12.89	68.94±40.45
Repeated measures analysis of variance							
Tests of Within-Subject Effects		Tests of Between-Subject Effects (P=0.001)					
t (P=0.022)	Time*group (P=0.006)						
t-test	rhG-CSF vs. control	P=0.994	P=0.559	P=0.050	P=0.069	P=0.000	P=0.044

Table 7. Changes in IgG count after rhG-CSF treatment ($\bar{x}\pm s$)

Group	n	0 d	2 d	5 d	7 d	11 d	14 d
rhG-CSF	20	0.67±0.18	0.51±0.18	1.10±0.19	1.37±0.13	1.72±0.37	1.78±0.17
Control	20	0.64±0.12	0.80±0.25	0.93±0.08	0.97±0.33	0.82±0.35	0.89±0.13
Repeated measures analysis of variance							
Tests of Within-Subject Effects		Tests of Between-Subject Effects (P=0.000)					
t (P=0.006)	Time*group (P=0.015)						
t-test	rhG-CSF vs. control	P=0.785	P=0.168	P=0.208	P=0.128	P=0.037	P=0.002

Table 8. Changes in IgM count after rhG-CSF treatment ($\bar{x}\pm s$)

Group	n	0 d	2 d	5 d	7 d	11 d	14 d
rhG-CSF	20	68.08±30.64	42.69±23.85	68.80±26.45	80.12±19.16	105.74±26.86	134.11±29.01
Control	20	62.29±6.93	62.98±10.90	38.57±21.05	54.05±21.47	26.12±11.04	39.78±21.93
Repeated measures analysis of variance							
Tests of Within-Subject Effects		Tests of Between-Subject Effects (P=0.001)					
t (P=0.022)	Time*group (P=0.006)						
t-test	rhG-CSF vs. control	P=0.766	P=0.251	P=0.196	P=0.192	P=0.009	P=0.011

methods between two groups ($P<0.01$). The difference in the expression level between the rhG-CSF and control group was statistically significant ($P<0.05$). The IgA expression level in the rhG-CSF group was significantly higher than that of the control group on the 11th and 14th day ($P<0.05$, **Table 6**).

The difference in the IgG expression level measured at different time was statistically significant ($P<0.01$) and it was affected by time and the different treatment methods between two groups ($P<0.05$). The difference in the IgG expression level between the rhG-CSF and control groups was statistically significant ($P<0.05$). The IgG expression level in rhG-CSF group was significantly higher than that of the control group on the 11th and 14th day ($P<0.05$, **Table 7**).

The difference in the IgM expression level measured at different time was statistically significant ($P<0.01$) in a time-dependent manner, as well as, affected by the different treatment methods between two groups ($P<0.05$). The difference in the IgM expression level between the rhG-CSF and control groups was statistically significant ($P<0.05$). The IgM expression level in the rhG-CSF group was significantly higher than that of the control group on the 11th and 14th day ($P<0.05$, **Table 8**).

These results suggested that rhG-CSF can increase the expression efficiency of IgA, IgG, and IgM, and increase the immunity of rabbits in the rhG-CSF group.

Bone marrow biopsy results

To investigate the change in bone marrow proliferation, bone marrow samples were examined. After injecting CTX for 4 days, the normal structure of bone marrow (**Figure 2A**) in rabbits was damaged, the hematopoietic tissue became less, and the adipose tissue formed more than before (**Figure 2B**). After rhG-CSF treatment, the hematopoietic tissue became more nearly the normal structure (**Figure 2C**).

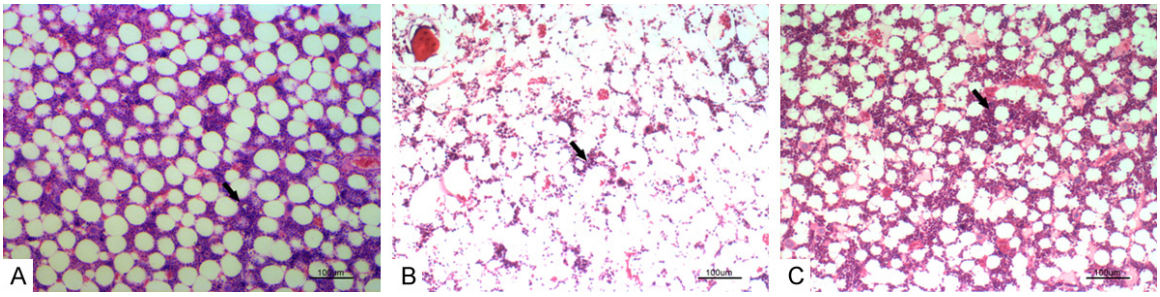

Figure 2. Bone marrow structure was stained by hematoxylin-eosin (magnification ×100). A. Normal structure of bone marrow. B. Injured bone marrow of the rabbit after injected CTX for 4 days. C. Recovered bone marrow structure after injecting rhG-CSF for 7 days from rhG-CSF group. (The black arrows indicate the hematopoietic tissue).

The results showed that CTX inhibited the bone marrow proliferation, while the rhG-CSF relieved the myelosuppression and improved the proliferation of bone marrow.

Discussion

Myelosuppression is a major side-effect of chemotherapy that increases morbidity/mortality and health-care costs [15]. Leukopenia resulted from myelosuppression shows an increased probability of infection and an extension of the chemotherapy course in clinic [10, 16]. Therefore, the method to prevent and relieve myelosuppression should be found. It is recognized that the key factors that ensure the successful completion of chemotherapy and improve the clinical curative effect include: the prevention and mitigation of myelosuppression after chemotherapy, promotion of the recovery of hematopoietic function, increasing the number of WBC, and reducing the probability of infection-induced diarrhea.

In this study, we established a model of leukopenia in New Zealand rabbits and use the rhG-CSF to treat leukopenia due to CTX. The results showed that rhG-CSF significantly decreased the rate of diarrhea and mortality in model rabbits by enhancing the number of WBC in peripheral blood. The results were consistent with those of Billan et al. [17]. In the current study, the number of leukocytes was rapidly increased after rhG-CSF treatment, peaked on day 5, and followed by a decline below the normal level after discontinuation of the treatment. This change in the trend of leukocytes had a negative correlation with diarrhea and death in rabbits. Therefore, we speculated that rhG-CSF enhances the immunity of rabbits by increasing the number of WBCs, thereby reducing the rate of diarrhea and mortality. The rhG-CSF could regulate the proliferation, differentiation, and maturation of neutrophils, and release them to the peripheral blood, thus the number of neutrophils in the peripheral blood increased. In the classification of WBC, we found that rhG-CSF mainly increased the neutrophils and lymphocytes, and the effect on the monocytes was relatively weak. This phenomenon was in agreement with the potential of rhG-CSF to stimulate the myeloid progenitor cell proliferation and promote the development and maturation of granulocytes [18].

The specific humoral immune response of the body is primarily regulated by antibodies secreted by B lymphocytes. The number of lymphocytes in the rhG-CSF group was found to increase and peak on day 2 ($5.95\pm1.86\times10^9$/L), followed by a decrease on day 5 that dropped below the normal level; the lower limit of observation up to 14 days was not higher than the normal value after the injection of rhG-CSF. The number of lymphocytes in the control group fluctuated below normal, and no obvious increase or decrease was observed. The expression level of Ig in rabbit serum was assessed, and the results showed that rhG-CSF could effectively increase the IgA, IgG, and IgM in serum. After treatment with rhG-CSF, the rates of diarrhea and mortality in rabbits in the rhG-CSF group were significantly lower than those in the control group, which might be attributed to the rhG-CSF-mediated increase in the number of lymphocytes and the expression level of IgA, IgG, and IgM. Consequently, the immunity of the model rabbits was enhanced, the immunosuppression caused by cyclophosphamide was alleviated early and rapidly, and the immune

defense reaction of the rabbits was strengthened. On the other hand, human Ig combines the Fc receptors in IgG and binds them on the lymphocytes for the anti-inflammatory, anti-infection, and immunomodulatory function. It can also regulate the inflammatory cells directly and control the spread of inflammation at the early stage [19]. These effects of immunoglobulin effectively reduced the rates of diarrhea and mortality in the model rabbits. Based on the results of lymphocytes and immunoglobulins, we concluded that rhG-CSF might exert an anti-infective effect by enhancing the humoral immunity of model rabbits, thereby reducing the rate of diarrhea and mortality.

In this study, the side effects on rhG-CSF-treated leukopenia were rarely observed and need further follow-up in future experiment. As only IgA, IgG, and IgM were detected but no lymphocyte classification was observed, the immunomodulatory effects of rhG-CSF on the decreased rate of diarrhea and mortality in model rabbits warrant further experimental confirmation.

In summary, rhG-CSF can reduce the incidence of infection, thus reducing the rate of diarrhea and mortality during the treatment of chemotherapy-induced diarrhea by increasing the number of neutrophils, lymphocytes, and immunoglobulins.

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Disclosure of conflict of interest

None.

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