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ORIGINAL ARTICLE

Protective Effect of 20(R)-ginsenoside Rg3 on Chemotherapy-induced Myelosuppression in Mice

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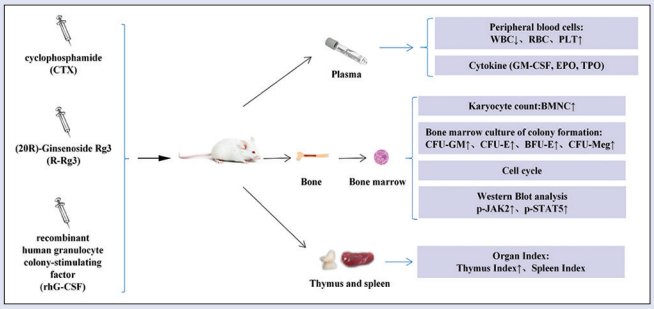
ABSTRACT

Background: 20(R)-ginsenoside Rg3 (R-Rg3) is a kind of ginseng glycol tetracyclic triterpenoid saponin, which is a recognized traditional Chinese medicine monomer and has the action of inhibiting the proliferation of tumor cells. **Objectives:** The goal of our experiment was to investigate the protective function of R-Rg3 on chemotherapy-induced myelosuppression in mice. **Materials and Methods:** Cyclophosphamide (CTX) was injected into the intraperitoneal (i.p.) to establish the mice myelosuppression model. We measured the number of peripheral blood cells (PBCs), and the number of bone marrow nucleated cells (BMNCs) was counted. Hematopoietic progenitor cells (HPCs) were cultured *in vitro*, and the amount of cell colonies was recorded at different times. Then ELISA was used to detect the levels of hematopoietic-related cytokines and flow cytometry was employed to test cell cycle. The network pharmacology was used to predict the main pathways of action. Furthermore, the expression of p-JAK2 and p-STAT5 was detected via western blotting. **Results:** The experimental outcomes showed that R-Rg3 could improve the amount of PBCs in mice with myelosuppression, increase the quantity of karyota cells in bone marrow, and enhance the proliferation of HPCs and adjust the content of hematopoietic-related cytokines. Moreover, the JAK–STAT signaling pathway may be the key to the role of R-Rg3. **Conclusion:** All of these results implied that R-Rg3 might be a therapeutic agent for myelosuppression after chemotherapy.

Key words: 20(R)-ginsenoside Rg3, chemotherapy, cyclophosphamide, myelosuppression, network pharmacology

SUMMARY

- The experiment was to investigate the protective function of R-Rg3 on chemotherapy-induced myelosuppression in mice. The method of intraperitoneal injection of CTX was used to establish the mice myelosuppression model. The numbers of PBCs and BMNCs were detected, and thymus and spleen indices were measured. Through examining the changes in hematopoietic-related cytokines and the cell cycle, we researched the impacts of drugs on the proliferation and differentiation of HPCs. The network pharmacology was used to predict R-Rg3's main action pathways, and the expression levels of p-JAK2 and p-STAT5 were tested. Finally, it was concluded that R-Rg3 could improve the quality of life of the myelosuppressed mice.



Abbreviations used: CTX: Cyclophosphamide; BMNCs: Bone marrow nucleated cells; HPCs: Hematopoietic progenitor cells; GM-CSF: Granulocyte-macrophage colony-stimulating factor; PBS: Phosphate buffer saline; CFU-E: Colony-forming unit erythroid; BFU-E: Burst-forming unit erythroid; CFU-GM: Colony-forming unit granulocyte macrophages; CFU-Meg: Colony-forming unit megakaryocyte; DAVID: Database for Annotation, Visualization and Integrated Discovery; KEGG: Kyoto Encyclopedia of Genes and Genomes; WBC: White blood cell; RBC: Red Blood Cell; PLT: Platelet.

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INTRODUCTION

Cancer has become a serious disease that harms human health in today's society. Chemotherapy is one of the commonly used treatments for malignant tumors at this stage. However, due to the lack of selectivity and more toxic side effects of chemotherapy drugs, myelosuppression often occurs after chemotherapy.[1] Cyclophosphamide (CTX) is the most widely used alkylating agent in clinical practice.[2] Relevant studies have shown that after the action of CTX, the division and proliferation of normal hematopoietic cells were blocked, resulting in the damage of bone marrow regeneration or hematopoietic system.[3] It can cause various symptoms such as anemia, hemorrhage and low immunity. It is prone to serious infections and even death,[4] which seriously affects the patient's quality of life. Therefore, the treatment of myelosuppression can greatly ameliorate the effect of chemotherapy and improve the quality of life of cancer patients.

Ginseng (*Panax ginseng* C. A. Mey.), which is a traditional Chinese herbal medicine in China, holds an important position in traditional Chinese

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medicine. Modern research reports that various active ingredients in ginseng, such as ginsenosides and ginseng polysaccharides, have pharmacological effects such as improving immunity, assisting tumor treatment, enhancing bone marrow hematopoietic function, and regulating the nervous system.[5–9] Ginseng also is the "Chinese medicine and food homologous" Chinese medicine approved by the Ministry of Health. Up to now, a variety of ginsenosides have been isolated and identified from ginseng, and each ginsenoside has its own physiological activity.[10] R-Rg3 is a kind of ginseng glycol tetracyclic triterpenoid saponin which is one of the main active components of ginseng. Its chemical composition is complex, has multi-target and multi-channel pharmacological activity, and has less toxicity than chemical drugs. It is recognized as a monomer of Chinese medicine with the effect of inhibiting the proliferation of tumor cells,[11,12] and also has many functions such as anti-diabetes,[13] anti-depression,[14] anti-inflammatory[15] effects, and so on. It has been developed and used all over the world, but no one has studied its therapeutic effect on myelosuppression.

In this study, we applied the method of intraperitoneal injection of CTX to establish the mice myelosuppression model. We detected the number of peripheral blood cells (PBCs) and bone marrow nucleated cells (BMNCs) in mice. After dissecting the mice, we measured their thymus and spleen index. In order to research the impacts of drugs on the proliferation and differentiation of hematopoietic progenitor cells (HPCs), we also carried out *in vitro* culture of HPCs. Additionally, we examined the changes in hematopoietic-related cytokines and cell cycle. Finally, network pharmacology was used to analyze the potential targets of R-Rg3 and predict its main action pathways, and the expression levels of p-JAK2 and p-STAT5 were tested.

MATERIALS AND METHODS

Materials

R-Rg3 was obtained from the Jilin University (Changchun, China). It was 99.5% pure, as confirmed by HPLC. Cyclophosphamide (CTX) was obtained from Baxter Oncology GmbH (Germany). Mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), thrombopoietin (TPO), erythropoietin (EPO) and interleukin-3 (IL-3) were purchased from Novoprotein Scientific Inc. (Shanghai, China).

Experimental Animal

Male Balb/c mice aged 6 to 8 weeks old were obtained from the Laboratory Animal Quality Testing Center of Jilin Province (Certificate no. SCXK-2016-0003). The mice were allowed to live in a temperature of 22 ± 2°C and humidity of 50 ± 10% with a 12 hr/12 hr light/dark cycle. All efforts were made to decrease the pain of the animals. The handling of animals was carried out in accordance with the rules of the National Institute of Health Laboratory Animal Care and Use Guidelines. And our research had been approved by the Animal Ethics Committee of the Chinese Academy of Sciences.[16]

After seven days of acclimatization, all mice were randomly divided into five groups (10 per group): (1) control group (control), (2) CTX treatment group (model), (3) rhG-CSF group (positive), (4) high-dose R-Rg3 treatment group (R-Rg3-H), and (5) light-dose R-Rg3 treatment group (R-Rg3-L). Except for the control group (given an equal amount of physiological saline 0.9% NaCl aq), the other groups were given CTX 100 mg/kg/d for 3 days. After modeling, the mice in group 3 were handled with rhG-CSF (11.25 μg/kg/d), the mice in groups 4 and 5 were handled with R-Rg3 (10 mg/kg/d, 5 mg/kg/d), the mice in groups 1 and 2 were handled with equivalent 0.9% NaCl aq for seven days.

Detection of peripheral blood cells

After the last administration of the drug for 24 hr, blood was collected from the orbital venous plexus. The number of blood cells was diluted and measured using a CX3 automatic biochemical analyzer.

Determination of the amount of karyota cells in bone marrow

Mice were euthanized by cervical dislocation and then immersed in 75% ethanol solution. Under sterile conditions, bilateral femurs were taken out and bone marrow cells were rinsed out with phosphate buffer saline (PBS). Then the single bone marrow cell suspension was prepared by filtration using a 4-gauge needle and centrifuged for 10 min at 1200 rpm. After taking out, the liquid was drained by using a pipette, and 0.5 mL of red blood cell (RBC) lysis buffer was added. After standing for 3 min, it was centrifuged for 10 min at 1200 r/min. Then the liquid was drained by using a pipette, the sediment was flushed twice with 1 mL PBS for 10 min at 1200 rpm and then resuspended in PBS. Next a pipette was use to drain the liquid and 1 mL of PBS was added to the mix. The number of bone marrow nucleus cells was calculated under an inverted microscope.

Determinations of thymus/spleen index

The mice were dissected to remove the thymus and spleen. After removing the adipose tissue, the weight was weighed using a precision electronic balance. The thymus and spleen index of the mice were calculated as follows:

Organ index (%) = (weight of organ/mouse body weight) ×100%

Hematopoietic progenitor cells culture

IMDM medium was plated with bone marrow cells at a concentration of 10^5/mL, then supplemented with horse serum, 10^{-4} M 2-mercaptoethanol, 2 mM 3% L-glutamine, 20 ng/mL (rm) IL-3, 20 U/mL (rh) EPO, 50 ng/mL (rm) GM-CSF, and 5 ng/mL (rm) TPO.

The culture environment of plates was 37°C with 5% CO_2. Colony-forming unit erythroid (CFU-E) was counted after 3 days of culture. Burst-forming unit erythroid (BFU-E), colony-forming unit granulocyte macrophages (CFU-GM), and colony-forming unit-megakaryocyte (CFU-Meg) were counted after 7 days of culture.[16]

Detection of cytokines in serum

Blood samples were taken under the 37°C water-bath for 30 min and centrifuged at 4000 rpm for 10 min to gain serum. After separating the serum, the concentrations of GM-CSF, EPO and TPO in the serum of each group were tested according to the enzyme-linked immunosorbent assay (ELISA) method.

Determination of the cell cycle

A single bone marrow cell suspension was centrifuged (1000 rpm, 10 min), then the supernatant was discarded. 2 mL 70% cold ethanol was added to fix the cells overnight at 4°C. The Propidium iodide dye (30 min avoid light) was added to stain. The flow cytometry was used for testing the changes in the cell cycle. The cell proliferation index (PI) was calculated according to the following formula:

$PI = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$

Network pharmacology

The main targets of R-Rg_3 were predicted and screened by Swiss Target Prediction database and UniProt database. Cytoscape 3.2.1 software was used to establish the "component-target" network. The Database

for Annotation, Visualization and Integrated Discovery (DAVID) was used for pathway prediction. The acquired pathways were enriched and analyzed through OmicShare database. The database for Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to further analyze the resulting pathways.

Western blotting analysis

The determination of protein content was done using western blot analysis. Bradford method was applied to determine protein concentration. SDS-PAGE electrophoresis was employed to separate denatured protein samples. Then the samples were transferred to PVDF membrane by semi-dry electrophoresis, closed in 5% skimmed milk liquid for 1 hr, and added primary antibody, 4°C overnight. After adding a secondary antibody (1:3000) and incubating for 1 hr, chemiluminescence was used to develop the color. β-actin was regarded as an internal reference. The protein bands were quantitatively analyzed, and the relative expression level of each target protein was calculated.[16]

Statistical analysis

All analyses were performed using the Statistical Pathway for the Social Sciences (SPSS) version 17.0 software, and experimental data were expressed as mean ± standard deviation. All data were analyzed by one-way analysis of variance (ANOVA). And $P < 0.05$ was considered to be a statistically significant difference.

RESULTS

Effects of R-Rg3 on WBC, RBC and PLT

Table 1 showed that contrasted with the control group, the number of white blood cells (WBCs) in the model group increased markedly ($P < 0.01$), and the number of RBC and PLT decreased significantly ($P < 0.01$). Contrasted with the number of WBCs in the model group, the R-Rg3-H group showed a significant decrease ($P < 0.01$), and there was no obvious difference in the R-Rg3-L group. There was no significant difference between the R-Rg3-L group and the R-Rg3-H group compared with the number of RBCs in the model group. Contrasted with the model group, the number of platelet (PLT) in the R-Rg3-L group and the R-Rg3-H group was markedly increased ($P < 0.01$).

Effect of R-Rg3 on the number of BMNCs

As shown in Figure 1, the number of BMNCs in the model group was markedly reduced in contrast to the control group ($P < 0.01$). Compared with the model group, the number of BMNCs in R-Rg3-L group and R-Rg3-H group was significantly increased ($P < 0.01$ or $P < 0.05$), and the effect of R-Rg3-H group was stronger than that in the R-Rg3-L group ($P < 0.01$).

Effects of R-Rg3 on thymus and spleen index

It can be seen from Table 2 that the thymus index of the model group decreased significantly ($P < 0.01$) and the spleen index increased significantly ($P < 0.01$) when compared with the control group. Compared with the model group, the thymus index of R-Rg3-L group and R-Rg3-H group were markedly increased ($P < 0.01$), and the effect of R-Rg3-H group was higher than that of R-Rg_3-L group ($P < 0.01$). The spleen index of R-Rg3-L group and R-Rg3-H group were lower than that of the model group, but the effects were not obvious.

Effects of R-Rg3 on colony yield of HPC *in vitro*

Figure 2 shows that the number of cell colonies of CFU-GM, CFU-E, BFU-E and CFU-Meg in the model group was markedly lower than that in the control group ($P < 0.01$). Compared with the model group, the number of cell colonies of CFU-GM, CFU-E, BFU-E and CFU-Meg in R-Rg3-H group was markedly higher ($P < 0.01$). Besides, the number of cell colonies of CFU-GM and CFU-Meg in R-Rg3-L group was markedly higher ($P < 0.05$). The colony numbers of R-Rg3-H group in CFU-GM, CFU-E, BFU-E and CFU-Meg were markedly higher than those in the R-Rg3-L group ($P < 0.01$).

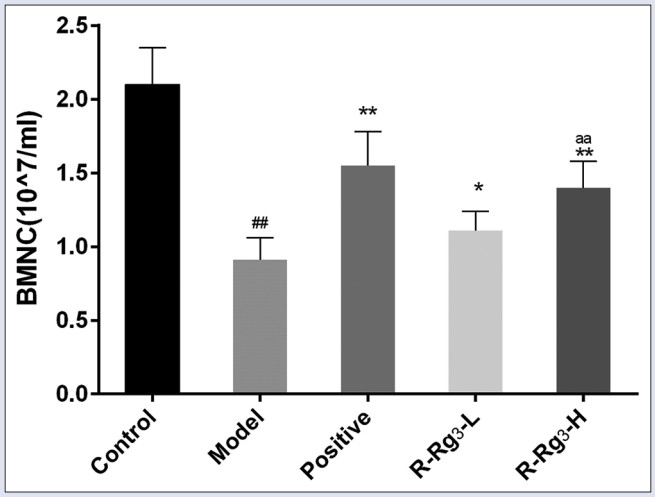

Figure 1: Effect of R-Rg3 on the number of BMNC ($n = 6$). ## $P < 0.01$ as compared with the control group. * $P < 0.05$ as compared with the model group. ** $P < 0.01$ as compared with the model group. aa $P < 0.01$ as compared with the R-Rg3-L group

Table 1: Effects of R-Rg3 on WBC, RBC and PLT

Groups	WBC (× 10^9/L)	RBC (× 10^{12}/L)	PLT (× 10^9/L)
Control	7.13±0.53	9.83±0.64	926.20±48.82
Model	10.08±1.12##	8.77±0.89##	631.40±66.83##
Positive	8.54±0.75**	9.51±0.66*	880.30±73.30**
R-Rg3-L	9.35±1.07	8.80±0.88	795.60±77.77**
R-Rg3-H	8.91±0.90**	9.18±0.89	848.40±78.25**

Data are expressed as mean±SD (n=10). ##P<0.01 as compared with the control group. *P<0.05 as compared with the Model group. **P<0.01 as compared with the Model group

Table 2: Effects of R-Rg3 on Thymus Index and Spleen Index

Groups	Thymus index (mg/kg)	Spleen index (mg/kg)
Control	1.21±0.14	3.34±0.40
Model	0.31±0.05##	8.80±1.30##
Positive	0.61±0.11**	8.11±1.00
R-Rg3-L	0.52±0.06**	8.67±0.97
R-Rg3-H	0.60±0.06**aa	8.46±0.76

Data are expressed as mean±SD (n=10). ##P<0.01 as compared with the control group. **P<0.01 as compared with the Model group. aa$P$<0.01 as compared with the R-Rg3-L group

Effects of R-Rg3 on hematopoietic-related cytokines

We measured the levels of hematopoietic-related cytokines GM-CSF, EPO and TPO. As shown in Figure 3, the levels of GM-CSF, EPO and TPO in the model group markedly increased compared with the control group ($P < 0.01$). The levels of GM-CSF and TPO in the R-Rg3-H group were significantly lower than those in the model group ($P < 0.01$ or $P < 0.05$). The levels of GM-CSF, EPO and TPO in the R-Rg3-L group were lower than those in the model group, but not obvious. The number of hematopoietic-related cytokines of R-Rg3-H group in GM-CSF and

TPO were markedly lower than those in the R-Rg3-L group ($P < 0.01$ or $P < 0.05$).

Effects of R-Rg3 on cell cycle

The distribution of each stage of the cell cycle is shown in Figure 4. In contrast with the control group, the bone marrow cell of mice in the model group were blocked in the G_0/G_1 phase, and the proliferation index (PI) of mice was reduced significantly ($P < 0.01$). The number of bone marrow cells in G_0/G_1 phase decreased, but that in G_2/M and S phases, it increased after R-Rg3 administration.

Results predicted by network pharmacology

Analysis of C-T network

R-Rg_3 was imported into the Swiss Target Prediction database to obtain potential targets (Top 15). Targets were imported into UniProt database for calibration. Cytoscape 3.2.1 software was used to construct the "component-target" (C-T) network, as shown in Figure 5, which contained 16 nodes (15 potential targets and 1 chemical components) and 15 edges.

Analysis of T-P network

Through the functional annotation tool in the DAVID, the potential targets of R-Rg3 were analyzed. Finally, a total of 7 pathways were obtained ($P < 0.05$). Cytoscape 3.2.1 software was employed to construct the target-pathway (T-P) network, as can be seen in Figure 6. Among them, there were 3 signal transduction pathways, 3 cancer-related pathways, and 1 immune disease-related pathway. Enrichment analysis of 7 pathways is shown in Figure 7. Among these 7 pathways, multiple pathways contained the JAK-STAT signaling pathway. Therefore, it was speculated that the JAK-STAT signaling pathway may be the key to the role of R-Rg3. The analysis methods were the same as the quoted article.[17]

Effects of R-Rg_3 on the expression of p-JAK2 and p-STAT5

Figure 8 shows the expression levels of p-JAK2 and p-STAT5 in different experimental groups. The expression levels of p-JAK2 and p-STAT5 in the model group significantly decreased ($P < 0.01$) when compared with the control group. Compared with the model group, the expression levels of p-JAK2 and p-STAT5 in the R-Rg3-H group significantly increased ($P < 0.01$).

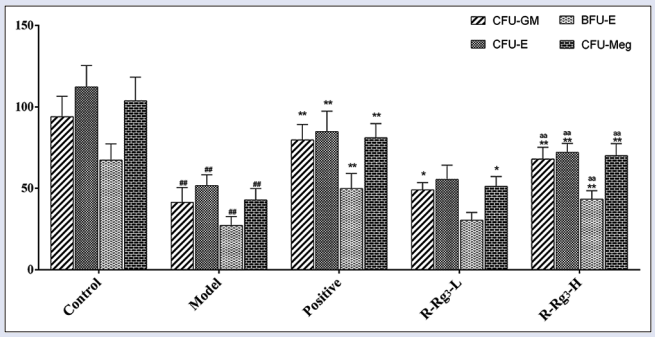

Figure 2: Effects of R-Rg_3 on cell colony number *in vitro* of hematopoietic progenitor cells ($n = 6$). ## $P < 0.01$ as compared with the control group. *$P < 0.05$ as compared with the model group. ** $P < 0.01$ as compared with the model group. aa $P < 0.01$ as compared with the R-Rg_3-L group

DISCUSSION

Chemotherapy is one of the first choices for the treatment of malignant tumors. However, chemotherapeutic drugs have the disadvantage of poor selectivity, and can cause killing effects on tumor cells and normal human cells. The side effects of bone marrow suppression are often the important reasons for patients not being able to complete chemotherapy on time.[18] Therefore, the presence of myelosuppression is one of the biggest obstacles in the treatment of cancer patients. By reducing the

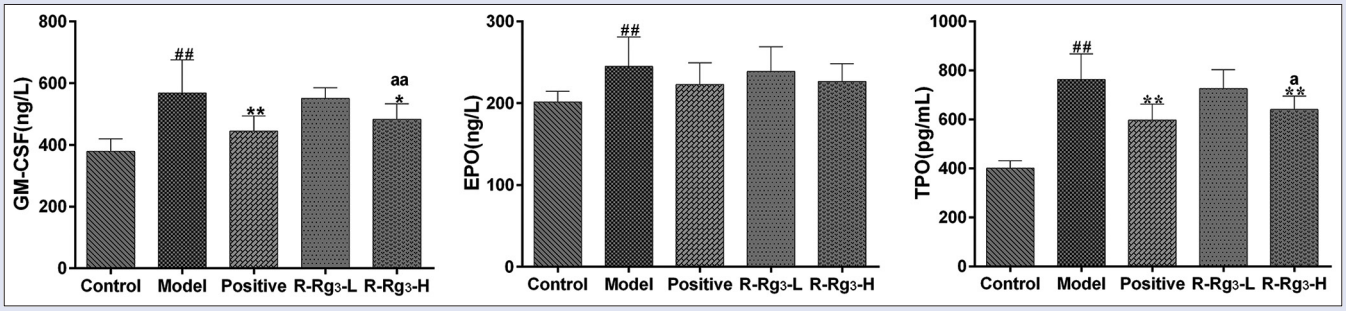

Figure 3: Effects of R-Rg_3 on hematopoietic-related cytokines ($n = 10$). ## $P < 0.01$ as compared with the control group. * $P < 0.05$ as compared with the model group. ** $P < 0.01$ as compared with the model group. aa $P < 0.01$ as compared with the R-Rg_3-L group. a $P < 0.05$ as compared with the R-Rg_3-L group

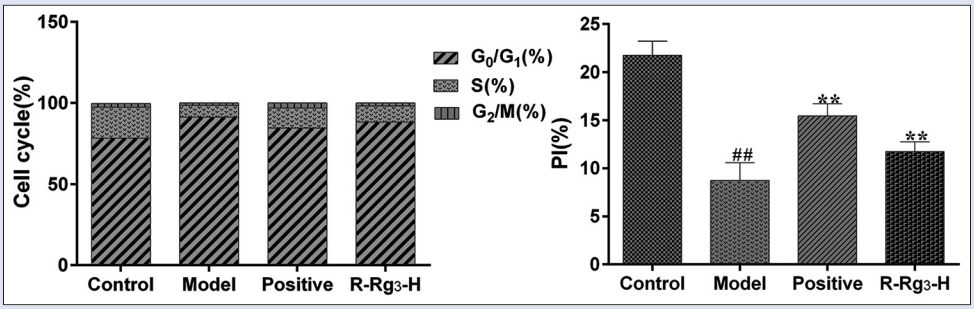

Figure 4: Effects of R-Rg_3 on cell cycle ($n = 6$). ## $P < 0.01$ as compared with the control group. ** $P < 0.01$ as compared with the model group

bone marrow suppression caused by chemotherapy and promoting the recovery of bone marrow hematopoietic function, the therapeutic effect of chemotherapy can be significantly improved, and the cancer patient's quality of life can be ameliorated.

In this study, we found that mice in the control group had better mental state, sensitive response, and increased body weight. The mice developed weight loss, poor mental state, lethargy, massive hair loss and reduced diet after intraperitoneal injection of CTX. After administration of the drug, the state of the mice recovered to varying degrees over time. Among them, the R-Rg3-H group recovered better than the R-Rg3-L group.

PBCs can indirectly reflect the hematopoietic function of the bone marrow, which plays an important role in maintaining life. WBCs are relatively large in volume and mainly play a role in phagocytosis. RBCs are the highest number of blood cells in the blood. It can transport oxygen to various tissues through hemoglobin. In addition, the carbon dioxide produced by the metabolism of various tissues in the body is also transported to the lungs through the RBCs and excreted. Platelets (PLTs) are biologically active, small cytoplasm whose main function is coagulation and hemostasis. The average survival time of WBCs is the shortest, RBCs is about 120 days and PLTs is about 5–7 days. Therefore, myelosuppression has the greatest impact on WBCs, and the effect on RBCs is usually small. In our research, the number of WBCs in the model group increased markedly. We speculated that this was a positive feedback effect of the body on the missing WBCs, resulting in a large number of differentiated WBCs released into the blood. However, these WBCs were incomplete or weakly immune, and the number of WBCs decrease rapidly after a brief increase, which was confirmed in our preliminary experiments. R-Rg3 could alleviate this mechanism to some extent. Additionally, R-Rg3 could significantly increase the amount of PLTs in the blood of myelosuppressed mice, which is very beneficial for the adjuvant treatment of chemotherapy.

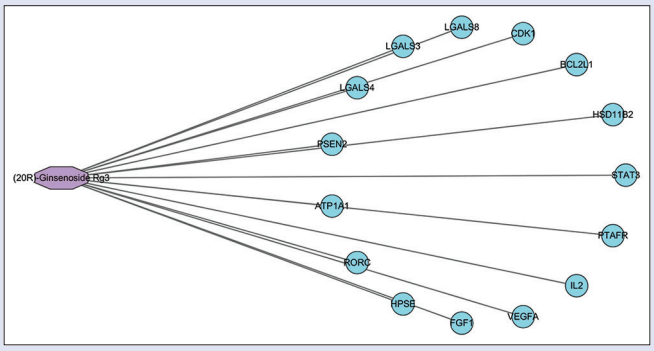

Figure 5: Compound-target network of R-Rg3

BMNCs can roughly represent the division and proliferation of bone marrow hematopoietic cells, which can directly reflect the hematopoietic function of bone marrow. The large number of BMNCs indicates that there are more immature blood cells, which reflects that the bone marrow has a better hematopoietic function.[19] The BMNC data of this experiment showed that R-Rg3 could significantly increase the number of BMNCs, which proved its good role in improving the myelosuppressed mice.

Thymus and spleen are two main immune organs. The thymus itself secretes thymosin as a mature organ of T cells and plays a key role in immune system.[20] The protection of the thymus can undoubtedly protect cellular immunity, humoral immunity and non-specific cellular immune function.[21] According to reports in the literature, CTX causes apoptosis in thymocytes.[22,23] The outcomes of our research showed that CTX caused severe atrophy of the thymus. R-Rg_3 could improve this phenomenon by increasing the thymus index, which reversed thymus atrophy. The spleen is the largest peripheral lymphoid organ in the human body. And it has many important immune active cells and immune cytokines. When the bone marrow function is impaired, the spleen can play a role in compensating for hematopoiesis.[24] Injection of CTX in our experiment caused splenomegaly, which we speculated was due to spleen compensatory hematopoiesis. R-Rg3 could alleviate splenomegaly by reducing spleen index.

During bone marrow hematopoietic stem cells cultured *in vitro*, the cells were stimulated by different colony-stimulating factors to differentiate into different colonies. The proliferation and differentiation ability of bone marrow hematopoietic cells are reflected by the production of these colonies.[25] Compared with the control group, the colony yields of CFU-GM, CFU-E, BFU-E and CFU-Meg in the model group were markedly reduced, which indicated that the ability of HPCs to proliferate and differentiate in myelosuppressed mice was severely impaired. However, R-Rg3 could stimulate the formation of CFU-GM, CFU-E, BFU-E and CFU-Meg cell colonies, which could promote the recovery of hematopoietic function in myelosuppressed mice. Among them, the effect of high dose was more obvious.

In order to explore the mechanism of R-Rg3, we examined the levels of hematopoietic-related cytokines. HPCs are stimulated by different cytokines to differentiate into various morphologically identifiable blood cells.[26] GM-CSF is a polypeptide growth factor, which has a broad-spectrum effect. Bone marrow stromal cells are important sites for secreting GM-CSF.[27,28] It binds to GM-CSF receptors and promotes various hematopoietic cell proliferation and differentiation. Erythropoietin (EPO) is mainly produced by the kidneys and is one of the main cytokines for promoting the production of erythroid blood cells in mammals. TPO is mainly produced by the liver and kidneys. It is recognized as a specific positive regulator of megakaryocyte system.[29,30] Our study found that the levels of GM-CSF, EPO and TPO in myelosuppressed mice increased, which should be the feedback

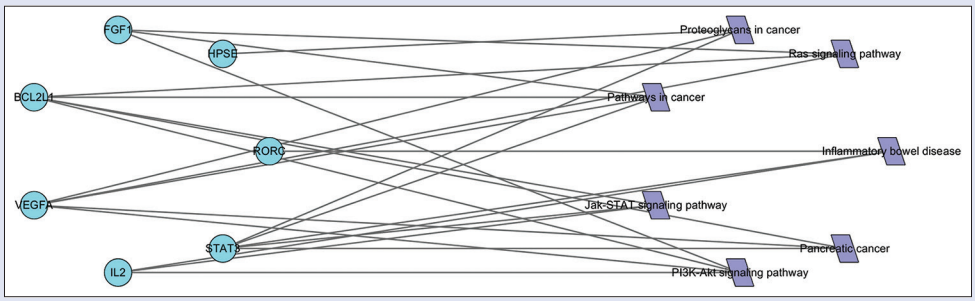

Figure 6: Target-pathway network of R-Rg3

mechanism of the body for various cell reduction. In addition, the results of the HPC culture showed that the ability of cell proliferation and differentiation of myelosuppressed mice had weakened. We speculated that hematopoietic progenitors are less sensitive to these hematopoietic factors, or hematopoietic progenitors are difficult to complete normal proliferation and differentiation after stimulation with these factors, and the body thus produces more cytokines. After treatment with R-Rg3, the ability of HPCs to proliferate and differentiate was improved, so the various hematopoietic cytokines secreted by the body were correspondingly reduced.

Cell proliferation is achieved through the operation of the cell cycle. The bone marrow cell cycle is the main indicator reflecting the hematopoietic action. The state of proliferation of the bone marrow cells can be reflected by the distribution of various phases of the cell cycle. There are two important checkpoints in the cell cycle regulation mechanism which are between G_1 and S, and G_2 and M.[31,32] The former is entering the DNA synthesis phase, and the latter is entering the mitosis phase.[25] In our experiment, after intraperitoneal injection of CTX, the percentage of G_0/G_1 phase cells in the model group was markedly higher than that in the control group, and the percentage of G_2/M phase cells was significantly lower than that in the control group. This suggested that CTX could cause G_1 phase arrest, which in turn led to S phase arrest. After treatment with R-Rg3, the percentage of G_0/G_1 cells was markedly lower than that of the model group, and the percentage of cells in G_2/M phase had significantly increased. This indicated that R-Rg3 can promote the completion of DNA synthesis and promote the cell into the mitosis stage, thereby enhancing the ability of cell proliferation and differentiation.

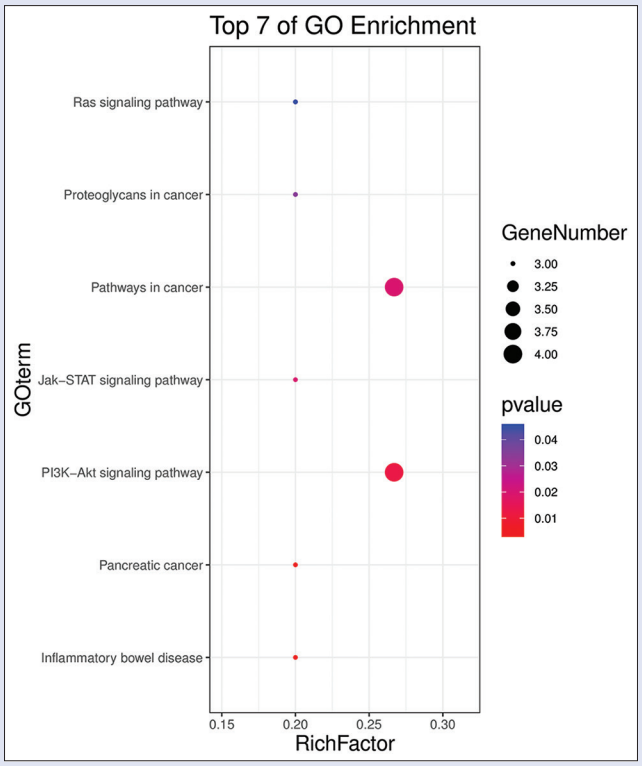

Figure 7: Pathway enriching of targets of R-Rg_3

JAK-STAT signaling pathway was first discovered in a study of signal molecules required for target gene activation after interferon action.[33] This signaling pathway directly transmits the received extracellular signals to the nuclear target gene promoter through a transmembrane receptor. It plays a very important regulatory role in the survival, proliferation, differentiation and apoptosis of hematopoietic cells.[34–36] JAK is a soluble cytosolic tyrosine protein kinase, and JAK2 is a member of the JAK family. It has seven domains, such as kinase homology domain 1, kinase homology domain 2 and amino-terminal FERM domain.[37] It plays an important role in cell survival and proliferation.[38] Existing studies have shown that the absence of JAK2 can cause severe HPC dysfunction and severe anemia in adult animals.[39] STAT is a direct substrate of JAK. It exists in the cytoplasm in a resting state. STAT5 is a member of the STAT family with seven domains.[40] A study by Lin *et al.*[41] found that if the STAT5 protein of mice was inhibited, the natural killer cells in the body were greatly reduced. Natural killer cells are important immune cells in the body. STAT5 maintains the survival and function of natural killer cells by promoting the expression of anti-apoptotic proteins and inhibiting the expression of pro-apoptotic proteins. A study by Wierenga ATJ *et al.*[42] found that STAT5 not only participated in hematopoietic differentiation, but also strengthened the self-renewal and erythroid differentiation of HPCs. Therefore, we speculated that the myelosuppression caused by CTX may be related to the inhibition of the JAK–STAT signaling pathway. Regulating the expression levels of JAK2 and STAT5 on the JAK–STAT signaling pathway may be one of the important mechanisms of R-Rg_3.

CONCLUSION

R-Rg3 could restore the number of blood cells, reverse thymus atrophy, relieve splenomegaly, enhance the proliferation and differentiation of bone marrow

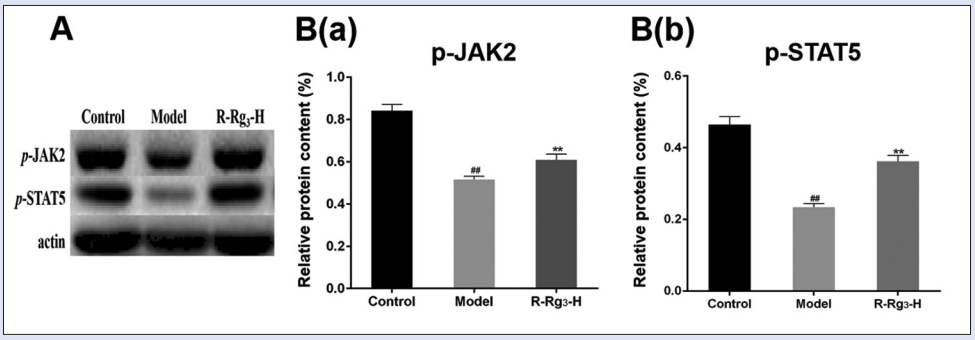

Figure 8: The expression levels of p-JAK2 and p-STAT5 in different experimental groups. (n = 3). (A) Use β-actin as an internal refere nce. After the film was scanned, the protein bands were quantitatively analyzed. (B (a)) The expression levels of p-JAK2 in different experimental groups. (B (b)) The expression levels of p-STAT5 in different experimental groups. ## $P < 0.01$ as compared with the control group. ** $P < 0.01$ as compared with the model group

hematopoietic stem cells, and promote the secretion of hematopoietic cell-associated cytokines, thereby improving the hematopoietic and immune functions of myelosuppressed mice to improve their quality of life.

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Conflicts of interest

There are no conflicts of interest.

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