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Research

High-altitude hypoxia exacerbates chemotherapy-induced myelosuppression by lowering serum G-CSF/GM-CSF and regulating apoptosis and proliferation

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Abstract

The unique hypoxic environment in high-altitude regions is increasingly drawing attention for its impact on the health of residents, particularly in patients post-chemotherapy. This study aimed to investigate the effects and potential mechanisms of high-altitude hypoxia on myelosuppression following chemotherapy, with the goal of providing a theoretical basis for clinical treatment. A retrospective clinical study of 80 patients with breast cancer revealed that patients in the plateau exhibited a significantly higher incidence of grade 3 or higher neutropenia and any level of neutropenia post-chemotherapy than those in the plain, with propensity score matching (PSM) confirming these associations. Animal experiments revealed that high-altitude hypoxia reduced the white blood cell (WBC) count, granulocyte count, lymphocyte count, and number of bone marrow nucleated cells (BMNCs) in cyclophosphamide (CTX)-treated mice. Additionally, high-altitude hypoxia induced a significant reduction in the proliferation index and an elevation in apoptosis rates in BMNCs. High-altitude hypoxia also significantly reduced serum levels of granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF). Transcriptomic analysis of BMNCs demonstrated that high-altitude hypoxia might modulate the hematopoietic function in CTX-induced myelosuppression mice through pathways related to hematopoiesis, such as porphyrin metabolism, hematopoietic cell lineage, ECM-receptor interaction, and PI3K-Akt signaling pathway. Our results suggest that high-altitude hypoxia exacerbates chemotherapy-induced myelosuppression, possibly through reducing the serum level of G-CSF/GM-CSF and regulating apoptosis and proliferation by PI3K-Akt signaling pathway, highlighting that cancer patients undergoing chemotherapy in hypoxic environments may require enhanced supportive care to mitigate these adverse effects.

Keywords High-altitude hypoxia · Chemotherapy · Myelosuppression · Hematopoietic function

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1 Introduction

High altitude is home to approximately 81.6 million people worldwide [1–3]. High altitudes are characterized by hypoxia, low pressure, low temperature, dry air, and strong ultraviolet radiation. Among these, hypoxia is the most significant factor affecting human physiology [4, 5]. The effects of high-altitude hypoxia on human physiological functions are complex and multifaceted. Reduced atmospheric pressure and lower oxygen partial pressures at high altitudes lead to insufficient oxygen intake, triggering a series of physiological and pathological responses [6]. Inadequate adaptation to high-altitude hypoxia can result in serious conditions such as pulmonary edema, stroke, cardiovascular dysfunction, and life-threatening situations [7]. However, these adaptations can also lead to health issues such as high-altitude polycythemia and chronic mountain sickness [8, 9].

It is widely known that hypoxic environments impact the hematopoietic system and can rapidly and persistently alter clinical blood parameters in humans [10–13]. However, most existing research has primarily focused on the impact of high-altitude hypoxia on hematopoiesis, particularly concerning red blood cell (RBC) and hemoglobin (Hb), with few studies investigating its effects on chemotherapy-induced myelosuppression. Myelosuppression is a common and serious side effect of chemotherapy that can lead to life-threatening complications such as infections, anemia, and bleeding [14–17]. In clinical practice, we have observed that the incidence of neutropenia, defined as an abnormally low level of neutrophil, appears to be higher in patients receiving chemotherapy at high altitudes compared to those in lowland areas. Consequently, the impact of high-altitude hypoxia on chemotherapy-induced myelosuppression remains an understudied area.

This study investigates the effects of high-altitude hypoxia on chemotherapy-induced neutropenia in patients with breast cancer. It also examines the impact of altitude hypoxia on chemotherapy-induced myelosuppression in mice and explores the underlying mechanisms.

2 Materials and methods

2.1 Patients and methods

The clinical portion of this study was an observational, retrospective, multicenter investigation involving patients treated at hospitals at different altitudes: Xining (2260 m), Qinghai Province, and Jining (37 m), Shandong Province. The inclusion criteria were female patients aged 18–75 years with a pathological diagnosis of breast cancer, who were treated with the EC regimen (epirubicin and cyclophosphamide) between October 2013 and December 2023, and had complete medical records. Patients also required no history of chemoradiotherapy, Karnofsky performance status (KPS) ≥ 80 points, and met the following laboratory parameters: Hb ≥ 95 g/L, white blood cell (WBC) count $\geq 3 \times 10^9$/L, and platelet (PLT) count $\geq 80 \times 10^9$/L. Exclusion criteria included the prophylactic use of granulocyte-stimulating factors after chemotherapy and the use of other chemotherapeutic regimens. Blood routine data were collected only after the first chemotherapy cycle and before the second cycle.

The study protocol and animal experiments were approved by the Ethics Committee of the Affiliated Hospital of Qinghai University (approval number P-SL-2023-447). Written informed consent was obtained from the patients prior to sample collection. All patient information was anonymized to ensure confidentiality. This research complies with the ethical standards outlined in the Declaration of Helsinki.

2.2 Animal experiments and drug treatments protocol

All procedures were conducted in strict accordance with the Guide for the Care and Use of Medical Laboratory Animals. Female C57BL/6 specific-pathogen-free (SPF) inbred mice, weighing 16–18 g, were sourced from Jiangsu Huachuang Xinnuo Pharmaceutical Technology Co., Ltd (Taizhou, China), under license number CNAS LA0021. The mice were housed in a controlled environment with temperatures between 24 °C and 26 °C, humidity levels of 50–60%, and a 12-h light/dark cycle. They were allowed a one-week acclimatization period with access to irradiated standard chow and sterilized purified water.

In this study, a total of 108 mice were used, of which 60 were used for blood routine tests within 21 days after chemotherapy, 24 were used for BMNCs counting, H&E staining, and transcriptomic sequencing, and 24 were used for the detection of cell cycle and apoptosis in BMNCs, as well as ELISA testing for hematopoietic cytokines. The mice were randomly assigned to one of four groups: control (ctrl), normoxic chemotherapy (model), hypoxia plus chemotherapy (HC), and hypoxia (H). Mice in the ctrl and model groups were housed in Xining (altitude: 2260 m, 16.2 kPa), Qinghai Province, while those in the HC and H groups were transported to Maduo County (altitude: 4300 m, PaO_2: 12.4 kPa), Qinghai Province, to facilitate comparative analysis of the effects of hypoxic conditions on the experimental outcomes. Mice in the HC and H groups were exposed to the hypoxic environment for the entire duration of the experiment, starting from their arrival in Maduo County until the completion of the study. This included a one-week acclimatization period prior to the initiation of any experimental procedures. Mice in the model and HC groups received intraperitoneal (i.p.) injections of cyclophosphamide (CTX) at a dose of 100 mg/kg for 3 days to develop the myelosuppressed mouse model, while the ctrl and H groups received an equal volume of saline.

All operations were conducted between 9:00 and 11:00 a.m. Blood samples, approximately 20 µL in volume, were collected from the mandibular veins on days 2, 7, 14, and 21 following the last drug administration. The mice used for the detection of hematopoietic cytokines were anesthetized with isoflurane, blood was collected via cardiac puncture, and then the mice were euthanized by cervical dislocation before they could recover from anesthesia. The other mice were euthanized by administering an intraperitoneal injection of pentobarbital at a dose of 200 mg/kg.

2.3 Cell counts in the peripheral blood

Peripheral blood samples were obtained from myelosuppressed mice on days 2, 7, 14, and 21 following the last CTX injection. Blood was collected via the mandibular vein and transferred into EDTA-coated tubes (cat no. YA1460; Solarbio Science and Technology Co. Ltd.) to prevent coagulation. Immediately after collection, a complete blood count was performed using a hematology analyzer (HKANGXING; China).

2.4 Isolation of bone marrow nucleated cells (BMNCs) and count

The experimental mice were euthanized, and the femoral bones were harvested immediately. Bone marrow components were flushed out using IMDM (cat no. PM150510; Procell Life Science and Technology Co., Ltd.) supplemented with 2% FBS (cat no. 10099141C; Gibco; Thermo Fisher Scientific, Inc.). A syringe equipped with a 21-gauge needle was used for this procedure. After passing through a 300-mesh strainer, BMNCs were isolated using red cell lysis buffer and then counted under a light microscope.

2.5 Cell cycle analysis

A single suspension of BMNCs ($5–10 \times 10^5$ cells) was collected in a centrifuge tube, and 1.5 mL of 70% cold ethanol was added to fix the cells overnight at − 20 °C. Following fixation, the cells were centrifuged at 300*g* for 5 min, and the supernatant was removed. The cells were then washed twice with PBS. Afterward, the cells were resuspended in 100 µL of RNase A Reagent from Cell Cycle Assay Kit (cat no. E-CK-A351, Elabscience Biotechnology Co.,Ltd.) at 37 °C for 30 min. Subsequently, staining was performed with 400 µL of propidium iodide (PI, 50 µg/mL) was performed in the dark at 2–8 °C for 30 min according to the manufacturer's instructions. Prior to analysis, the stained cells were filtered through a 70-µm cell strainer to ensure a single-cell suspension. Finally, the stained cells were analyzed with a BD FACSCelesta Multicolor Flow Cytometer (BD Biosciences) and the data were analyzed with FlowJo software.

2.6 Apoptosis analysis by annexin V/PI staining

BMNCs containing $5–10 \times 10^5$ cells were resuspended in 100 µL of the binding buffer from the apoptosis detection kit (cat no. E-CK-A211, Elabscience Biotechnology Co.,Ltd.). The cells were incubated with Annexin V-FITC and propidium iodide (PI) in the dark at room temperature for 15–20 min. After incubation, 400 µL of binding buffer was added to dilute the cells. Cell apoptosis was subsequently detected using a BD FACSCelesta Multicolor Flow Cytometer (BD Biosciences) and analyzed with FlowJo software.

2.7 Histopathological analysis

Collected femurs were fixed in a neutral buffered 10% formalin solution for 48 h to preserve the original morphological structure of the cells. Subsequently, the tissues were embedded in paraffin and sliced to a thickness of 4 μm. Notably, the bones were decalcified for 7 days prior to embedding. All slides were analyzed using hematoxylin and eosin (H&E) staining. After staining, the sections were placed in a TissueFAXS PLUS microscope (TissueGnostics, Vienna, Austria) for panoramic scanning, and the TissueFAXS Viewer software was utilized for image acquisition.

2.8 Enzyme-linked immunosorbent assay (ELISA)

The blood samples of mice were collected on day 2 post-chemotherapy, kept overnight at 4 °C, and then centrifuged at 3000*g*/min for 10 min to isolate serum. Commercial ELISA kits were utilized for the evaluation of Granulocyte-Colony Stimulating Factor (G-CSF) (cat no. KE10025; proteintech group Inc.), Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF) (cat no. MM-0185M1; Jiangsu Meimian industrial Co., Ltd), Erythropoietin (EPO) (cat no. MM-0194M1; Jiangsu Meimian industrial Co., Ltd), and Thrombopoietin (TPO) (cat no. MM-0194M1; Jiangsu Meimian industrial Co., Ltd). The ELISA assays were performed according to the manufacturers' instructions. The absorbance was measured at the wavelength of 450 nm using Cytation™5 (BioTek, USA).

2.9 RNA extraction and sequencing

Total RNA from BMNCs was extracted using TRIzol reagent (cat no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.) and subsequently stored at − 80 °C. Once all samples were prepared, they were sent to Shanghai Jiayin Biotechnology Ltd. for mRNA preparation and RNA sequencing (RNA-seq). Sequencing was performed using a Novaseq platform. The original sequencing data were deposited in the BioProject database (Bioproject ID PRJNA1176344).

2.10 RNA-seq data analysis

Differentially expressed genes (DEGs) were defined as genes with a fold change (FC) > 1 and *P* values < 0.05. Xiantao tool (www.xiantaozi.com/), an online bioinformatics analysis platform, was utilized to create a Venn diagram that illustrates the common and unique DEGs among the groups. Functional enrichment analysis was conducted using the Xiantao tool, generating functional processing categories and pathway enrichment based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, with an adjusted *P* value < 0.05. The "ClusterProfiler" (v4.4.4) R package [18] was used for the functional annotation and Gene Set Enrichment Analysis (GSEA) of the DEGs. The curated reference genesets from the MSigDB file: c2.cp.all.v2022.1.Hs.symbols.gmt were selected for GSEA [19]. Significantly enriched clusters were identified based on false discovery rate (FDR) < 0.25 and *P* (adjust) < 0.05 as threshold parameters.

2.11 Propensity score matching (PSM)

PSM technique represents an advanced and refined statistical approach for adjusting potential baseline confounders. We employed the MatchIt package in R 4.4.1 to execute a 1:1 match based on the nearest neighbor algorithm. The matched covariates included age, T stage, and N stage.

2.12 Statistical analysis

Pearson's chi-square test or Fisher's exact test is used to compare the incidence between the plateau and plain groups. Animal experimental data are presented as the mean ± standard deviation (SD). The comparison of the means between two samples was analyzed using the Student's *t* test, while comparisons among multiple samples were conducted using one-way ANOVA followed by Tukey's post-hoc test (GraphPad Prism 9.5.0). Two-way ANOVA was used for repeated

measurements followed by post-hoc analyses using the LSD test (SPSS 28.0). $P < 0.05$ was considered statistically significant.

3 Result

3.1 Effect of high-altitude hypoxia on neutrophil counts in patients with breast cancer undergoing chemotherapy

The study involved 80 eligible patients: 52 (65%) from a plain hospital (plain group) and 28 (35%) from a plateau hospital (plateau group). There were no significant differences in baseline characteristics between the two groups, including age, menstrual status, hormone receptor (HR) status, HER-2 status, tumor T stage, pathological stage, lymph node status, pathological staging, and histological type (Table 1). The incidence of neutropenia after the first cycle of chemotherapy was compared between the plateau and plain groups. The results indicated a significant incidence of grade 3 or higher neutropenia, with 30 patients (57.69%) in the plain group and 25 patients (89.29%) in the plateau group affected ($P = 0.004$). Additionally, 38 patients (73.08%) in the plain group and 27 patients (96.43%) in the plateau group exhibited some level of myelosuppression, with this difference also proving to be statistically significant ($P = 0.011$) (Table 2). To mitigate confounding bias and optimize statistical power, we employed Propensity Score Matching (PSM) to adjust for potential confounders and achieve balance in patient characteristics between the plateau and plain groups. Following PSM, each group consisted of 28 patients. Baseline characteristics of those patients are summarized in Table 1. There

Table 1 Baseline characteristics before and after propensity score matching

Characteristics	Before matching (n = 80)			After matching (n = 56)		
	Plain, n = 52	Plateau, n = 28	*P*	Plain, n = 28	Plateau, n = 28	*P*
Age			0.77			0.45
< = 60	44 (84.62%)	23 (82.14%)		25 (89.29%)	23 (82.14%)	
> 60	8 (15.38%)	5 (17.86%)		3 (10.71%)	5 (17.86%)	
Menopause			0.06			0.28
No	32 (61.54%)	11 (39.29%)		15 (53.57%)	11 (39.29%)	
Yes	20 (38.46%)	17 (60.71%)		13 (46.43%)	17 (60.71%)	
HR status			0.07			0.06
Positive	35 (67.31%)	24 (85.71%)		18 (64.29%)	24 (85.71%)	
Negative	17 (32.69%)	4 (14.29%)		10 (35.71%)	4 (14.29%)	
Her-2 status			0.36			0.24
Positive	6 (11.54%)	3 (10.71%)		5 (17.86%)	3 (10.71%)	
Negative	39 (75%)	24 (85.71%)		19 (67.86%)	24 (85.71%)	
Indeterminate	7 (13.46%)	1 (3.57%)		4 (14.29%)	1 (3.57%)	
T stage			0.4			0.75
T1	22 (42.31%)	9 (32.14%)		11 (39.29%)	9 (32.14%)	
T2	29 (55.77%)	17 (60.71%)		16 (57.14%)	17 (60.71%)	
T3 and T4	1 (1.92%)	2 (7.14%)		1 (3.57%)	2 (7.14%)	
N stage			0.36			0.12
N0	24 (46.15%)	10 (35.71%)		10 (35.71%)	10 (35.71%)	
N1	17 (32.69%)	8 (28.57%)		14 (50.00%)	8 (28.57%)	
N2 and N3	11 (21.15%)	10 (35.71%)		4 (14.29%)	10 (35.71%)	
Pathological staging			0.55			0.15
I	12 (23.08%)	6 (21.43%)		4 (14.29%)	6 (21.43%)	
II	29 (55.77%)	13 (46.43%)		20 (71.43%)	13 (46.43%)	
III	11 (21.15%)	9 (32.14%)		4 (14.29%)	9 (32.14%)	
Histotype			0.18			0.28
Ductal	40 (76.92%)	25 (89.29%)		22 (78.57%)	25 (89.29%)	
Other	12 (23.08%)	3 (10.71%)		6 (21.43%)	3 (10.71%)	

Table 2 Incidence of neutropenia in patients with breast cancer undergoing chemotherapy at varying altitudes

	Plain group, n = 52	Plateau group, n = 28	*P*
Grade 3 or higher neutropenia, n (%)	30 (57.69)	25 (89.29)	**0.004**
Any level of neutropenia, n (%)	38 (73.08)	27 (96.43)	**0.011**

were no significant differences in baseline characteristics between the two groups. The incidence of neutropenia after the first cycle of chemotherapy was compared between plateau and plain groups. The results indicated a significant incidence of grade 3 or higher neutropenia, with 17 patients (60.71%) in the plain group and 25 patients (89.29%) in the plateau group affected ($P = 0.014$). Additionally, 21 patients (75.00%) in the plain group and 27 patients (96.43%) in the plateau group exhibited some level of myelosuppression, with this difference also proving to be statistically significant ($P = 0.022$) (Table 3). These findings suggest that high-altitude hypoxia may play a critical role in influencing the incidence of neutropenia in patients with breast cancer undergoing chemotherapy.

3.2 Effect of high-altitude hypoxia on peripheral blood cell counts in CTX-induced myelosuppressive mice

A complete blood count was performed on days 2, 7, 14, and 21 after the last cyclophosphamide (CTX) injection (Fig. 1A). The study included four experimental groups: control (ctrl) group, normoxic chemotherapy (model) group, hypoxia plus chemotherapy (HC) group, and hypoxia (H) group. As shown in Fig. 1B–G, the white blood cell (WBC), granulocyte and lymphocyte and red blood cells (RBC) counts in the model group were significantly lower than those in the ctrl group on day 2 after chemotherapy ($P < 0.01$), which suggests that myelosuppression took place in the mice. The WBC, granulocyte, lymphocyte counts decreased significantly ($P < 0.05$) in the HC group when compared with the model group on day 2, 7, 21 after chemotherapy and the lowest value occured on day 2 after chemotherapy. The platelet (PLT) count decreased significantly ($P < 0.05$) in the HC group when compared with the model group on day 2, 14, 21 after chemotherapy. However, RBC counts and hemoglobin (Hb) level in the HC group were significantly higher than those in the model group ($P < 0.05$) on day 2, 7, 21 after chemotherapy. Meanwhile, RBC counts and Hb level in the H group were significantly higher than those in the ctrl group at any time point. During chemotherapy, WBC, granulocyte, and lymphocyte counts decreased significantly in the model group, with a more pronounced reduction observed in the HC group. While RBC and Hb levels also decreased significantly in the model group, the reduction was less pronounced in the HC group. The adverse effects of altitude hypoxia on myelosuppression mainly occurred in leukocytes, namely granulocytes and lymphocytes. So, we chose 2 days after chemotherapy as the time node of the follow-up experiment.

3.3 High-altitude hypoxia aggravates impaired hematopoiesis in CTX-induced myelosuppressive mice

For bone marrow nucleated cells (BMNCs) count and bone marrow hematoxylin and eosin (H&E) staining, mouse femurs were harvested 2 days after chemotherapy (Fig. 2A). CTX significantly reduced the BMNCs count ($P < 0.05$), with an even greater reduction observed in the HC group ($P < 0.05$) (Fig. 2B). H&E staining of femurs demonstrated notable bone marrow cell depletion in CTX-treated mice (Fig. 2C). The H&E staining revealed that in the ctrl group, the bone marrow structure appeared normal, with all types of hematopoietic cells evenly distributed within rich hematopoietic zones, and mature erythrocytes present in the sinusoids. Conversely, in the model group, there were few nucleated cells in the marrow cavities of femurs, while the hemorrhagic changes in the HC group were more severe than those in the model group. These observations suggest that high-altitude hypoxia, in the context of CTX treatment, may result in impaired

Table 3 Incidence of neutropenia in patients with breast cancer undergoing chemotherapy at varying altitudes after propensity score matching

	Plain group, n = 28	Plateau group, n = 28	*P*
Grade 3 or higher neutropenia, n (%)	17 (60.71)	25 (89.29)	**0.014**
Any level of neutropenia, n (%)	21 (75.00)	27 (96.43)	**0.022**

angiogenesis, increased hemorrhage, and further reductions in BMNC counts, which requires further investigation and validation.

3.4 RNA-seq identifies transcriptomic profiles of high-altitude hypoxia in BMNCs of CTX-induced myelosuppressive mice

To explore the mechanism of the effect of altitude hypoxia on bone marrow suppression, we used CTX to establish a myelosuppression model by intraperitoneal injection that continued for 3 days. After confirming the decreased hematopoietic function resulting from CTX treatment through WBC count on day 2 post-chemotherapy, transcriptome profiling analyses of BMNCs in the ctrl group, model group, and HC group were conducted to elucidate the underlying mechanisms. By applying the cutoffs of P value < 0.05 and FC > 1, the differentially expressed genes (DEGs) were selected. Compared to the ctrl group, 927 genes were upregulated, and 882 genes were downregulated in the model group (Fig. 3A, B). Compared to the model group, 130 genes were upregulated, and 550 genes were downregulated in the HC group (Fig. 3A and C). Compared to the ctrl group, 183 genes were upregulated, and 388 genes were downregulated in the H group (Fig. 3A and D).

Following this, we analyzed the co-regulated DEGs between the HC group vs the model group and the model group vs the ctrl group. The Venn diagram showed that 224 co-regulated DEGs were commonly affected by both high-altitude hypoxia and chemotherapy treatment and (Fig. 3E). Hierarchical clustering analysis showed that of the 224 DEGs, most of the co-regulated DEGs were consistent down-regulation (Fig. 3G). Finally, we explored the co-regulated DEGs between the HC group vs the model group and the H group vs the ctrl group. The Venn diagram showed that 146 co-regulated DEGs were related to hypoxia (Fig. 3F). Hierarchical clustering analysis showed that most of the co-regulated DEGs were also uniformly downregulated (Fig. 3H).

3.5 Functional enrichment analysis reveals key pathways regulated by high-altitude hypoxia in CTX-induced myelosuppressive mice

In order to further explore the potential mechanism of high altitude hypoxia affecting myelosuppression after chemotherapy, we conducted pathway enrichment analysis. GO analysis suggested that these DEGs related to high-altitude hypoxia between HC and model groups were classified into 961 different biological process categories, the top 10 of which encompassing erythrocyte development, B cell receptor signaling pathway, phagocytosis, recognition, respiratory system development, complement activation, B cell activation, homeostasis of number of cells, respiratory tube development, complement activation classical pathway, and regulation of B cell activation (Fig. 4A). The results supported the assumption that high-altitude hypoxia may have regulatory effect on the hematopoietic process during chemotherapy-induced myelosuppression.

KEGG enrichment analysis of DEGs between the HC and the model groups enriched 10 pathways, such as PI3K-Akt signaling pathway, focal adhesion, hematopoietic cell lineage, and ECM-receptor interaction (Fig. 4B).

GSEA showed that DEGs associated with hypoxia significantly enriched in cell cycle and DNA replication processes-related clusters, including cell cycle checkpoints, mitotic G1 phase and G1 S transition, DNA replication, cell cycle mitotic, and G2 M checkpoints, and the top 10 terms are shown in Fig. 4C. DEGs also showed significant correlation with the apoptosis (Fig. 4D).

Through enrichment analysis, we hypothesize that high-altitude hypoxia exacerbates chemotherapy-induced myelosuppression, possibly via regulation of cell cycle and apoptosis in BMNCs by multiple hematopoiesis-related pathways, such as PI3K-Akt signaling pathway and ECM-receptor interaction. To test this hypothesis, we conducted a follow-up experiment.

3.6 Effects of high-altitude hypoxia on the cell cycle of BMNC in CTX-induced myelosuppressive mice

In the model group, the proportion of cells in the G0/G1 phase significantly decreased compared to the ctrl group ($P < 0.05$), while the proportions of cells in the S and G2/M phases increased ($P < 0.05$). The proliferation index (PI) was significantly higher than in the ctrl group ($P < 0.05$). These results suggest that CTX-induced DNA damage triggered cell cycle arrest at the G2/M checkpoint. In contrast, the HC group showed a higher proportion of cells in the G0/G1 phase and lower proportions in the S and G2/M phases compared to the model group ($P < 0.05$). The PI was significantly lower in the HC group than in the model group ($P < 0.05$), indicating that hypoxia suppressed cell cycle progression and induced

Fig. 1 Effect of high-altitude hypoxia on peripheral blood cell counts in CTX-induced myelosuppression mice. Mice were administered CTX ▶ at a dose of 100 mg/(kg d) via intraperitoneal injection for 3 consecutive days, while the control (ctrl) group was treated with the same volume of saline. **A** Experimental design. The levels of WBC (**B**), granulocyte (**C**), RBC (**D**), Hb (**E**), PLT (**F**), and lymphocyte (**G**) were assessed on days 2, 7, 14, and 21 post-chemotherapy. *WBC* white blood cell, *Gran* granulocyte, *RBC* red blood cell, *Hb* hemoglobin, *PLT* platelets, *Lym* lymphocyte. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to the ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to the model group. n = 15

G1 phase arrest. In the H group, the proportion of cells in the G0/G1 phase showed no significant difference compared to the ctrl group ($P > 0.05$), while the proportions of cells in the S and G2/M phases were significantly increased ($P < 0.05$). The PI was also significantly higher than in the ctrl group ($P < 0.05$). These results suggest that hypoxia alone promoted cell cycle progression, leading to an increase in cells entering the S and G2/M phases and enhancing proliferative activity.

3.7 Effects of high-altitude hypoxia on the apoptosis of BMNC in CTX-induced myelosuppressive mice

The apoptosis of BMNCs was assessed on day 2 post-chemotherapy, with results shown in Fig. 5C, D. Compared to the ctrl group, the model group exhibited a significant increase in apoptosis ($P < 0.05$), confirming that chemotherapy led to elevated levels of bone marrow cell apoptosis. Notably, the HC group, which underwent chemotherapy under hypoxic conditions, showed an even higher apoptosis rate, significantly surpassing that of the model group ($P < 0.05$).

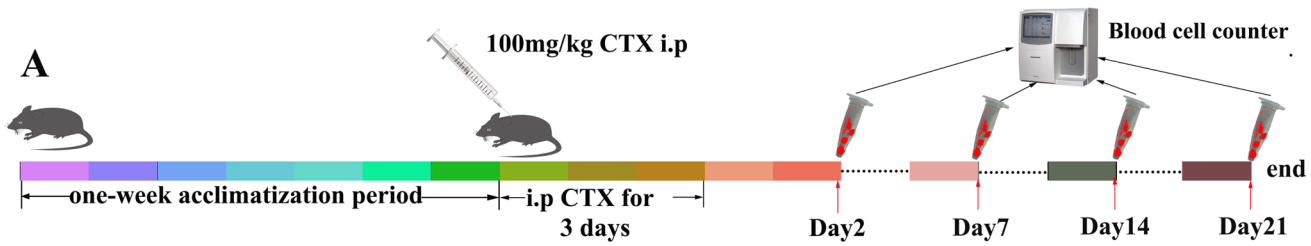
3.8 Effect of high-altitude hypoxia on the hematopoiesis-related cytokines in CTX-induced myelosuppressive mice

As illustrated in Fig. 6A–C, the levels of G-CSF, GM-CSF and EPO in the model group were significantly elevated compared to the ctrl group ($P < 0.05$). Hypoxia resulted in a significant reduction in G-CSF and GM-CSF levels in the HC group compared to the model group ($P < 0.001$), while it significantly increased EPO levels ($P < 0.001$). No statistically significant differences were observed in TPO levels among the four groups (Fig. 6D).

4 Discussion

This study investigates the impact of high-altitude hypoxia on hematopoietic function through both clinical analysis and an experimental mouse model of CTX-induced myelosuppression. Patients with breast cancer residing at high altitudes exhibited a significantly higher incidence of grade 3 or higher neutropenia and any level of neutropenia after chemotherapy. Experimental results indicated that high-altitude hypoxia during chemotherapy further reduced WBC, granulocyte, lymphocyte, and PLT counts while unexpectedly increasing RBC counts and Hb level. Additionally, high-altitude hypoxia increased cell apoptosis, inhibited cell proliferation, decreased G-CSF and GM-CSF levels and increased EPO levels in the CTX-induced myelosuppressive mice. RNA-seq analysis revealed several hematopoiesis-related signaling pathways influenced by hypoxia, underscoring the complexity of its effects on bone marrow function.

Cyclophosphamide (CTX), a classic alkylating agent, is widely used in the chemotherapy of various malignant tumors. Its anti-tumor mechanism primarily relies on its ability to damage DNA, especially affecting rapidly proliferating cells. The main mechanism of action of cyclophosphamide involves its metabolites forming cross-links with DNA, leading to DNA strand breaks and damage. This DNA damage activates the cell's repair mechanisms, but if the damage is too severe, the cell may enter the programmed cell death (apoptosis) pathway, resulting in cell death [20]. The alkylating effect of cyclophosphamide is not limited to tumor cells; it also affects the DNA of normal cells, leading to myelosuppression and reproductive toxicity, which are among the main side effects in its clinical application [21]. Additionally, the impact of CTX on the cell cycle is mainly reflected in its induction of cell cycle arrest and apoptosis. Cyclophosphamide can cause cells to arrest in the G0/G1 or G2/M phase, preventing them from entering the next proliferative phase. This arrest is due to the DNA damage caused by cyclophosphamide, which activates cell cycle checkpoints, particularly the p53 signaling pathway, leading to changes in the expression of cell cycle regulatory proteins and thereby affecting the cell's proliferative capacity [22]. In tumor cells treated with cyclophosphamide, the proportion of cells in the G2/M phase significantly increases, while the proportion of cells in the G0/G1 phase decreases, indicating that cyclophosphamide can effectively inhibit cell proliferation [23]. Additionally, cyclophosphamide promotes cell death by inducing the expression of apoptosis-related proteins, further enhancing its anti-tumor effects [20]. Cyclophosphamide exhibits significant immunosuppressive effects, primarily through the inhibition of lymphocyte proliferation and function. Studies have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100mg/kg CTX i.p
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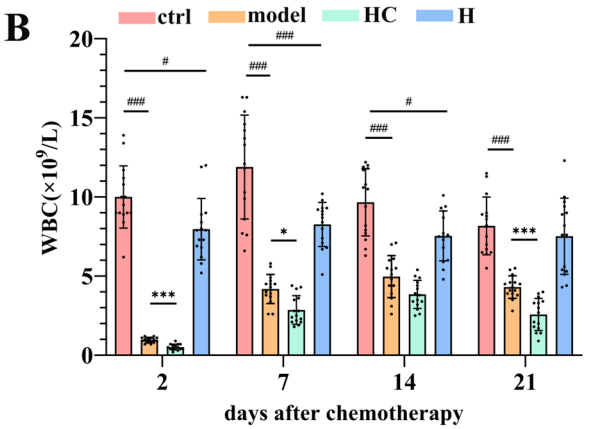
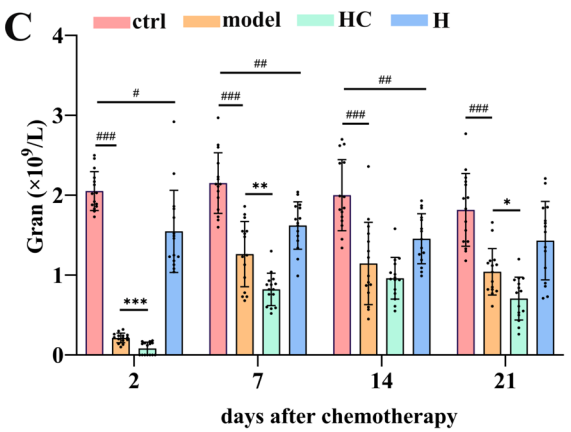
Day14
Day21
end


B
ctrl
model
HC
H
WBC(×10^9/L)
days after chemotherapy


C
ctrl
model
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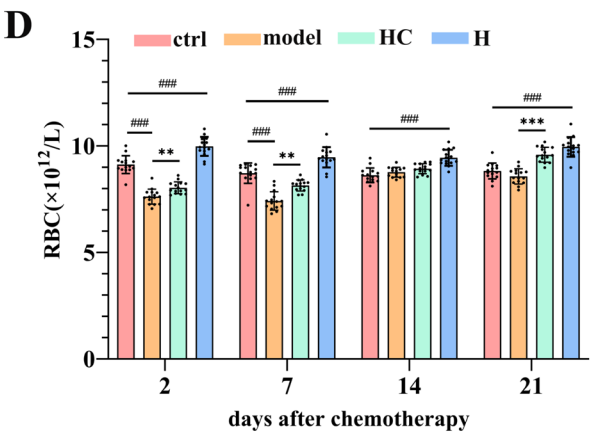
H
Gran (×10^9/L)
days after chemotherapy


D
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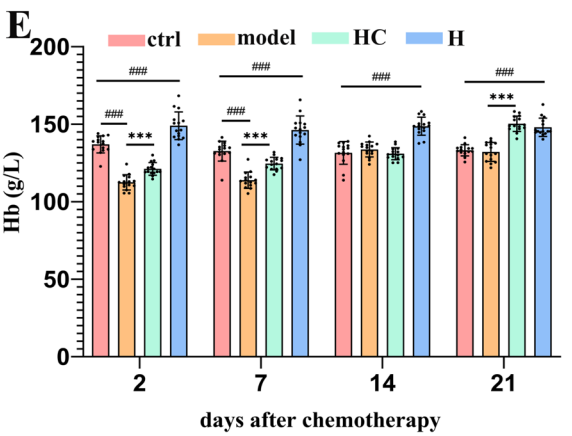
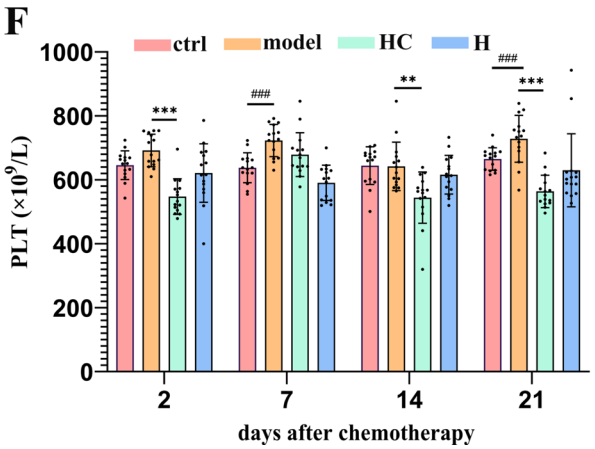
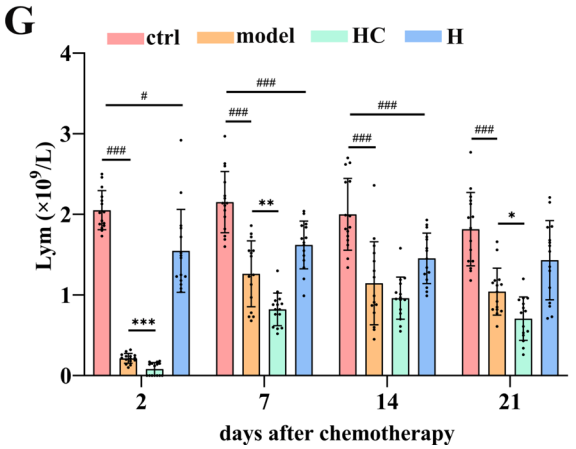
RBC(×10^12/L)
days after chemotherapy


E
ctrl
model
HC
H
Hb (g/L)
days after chemotherapy


F
ctrl
model
HC
H
PLT (×10^9/L)
days after chemotherapy


G
ctrl
model
HC
H
Lym (×10^9/L)
days after chemotherapy

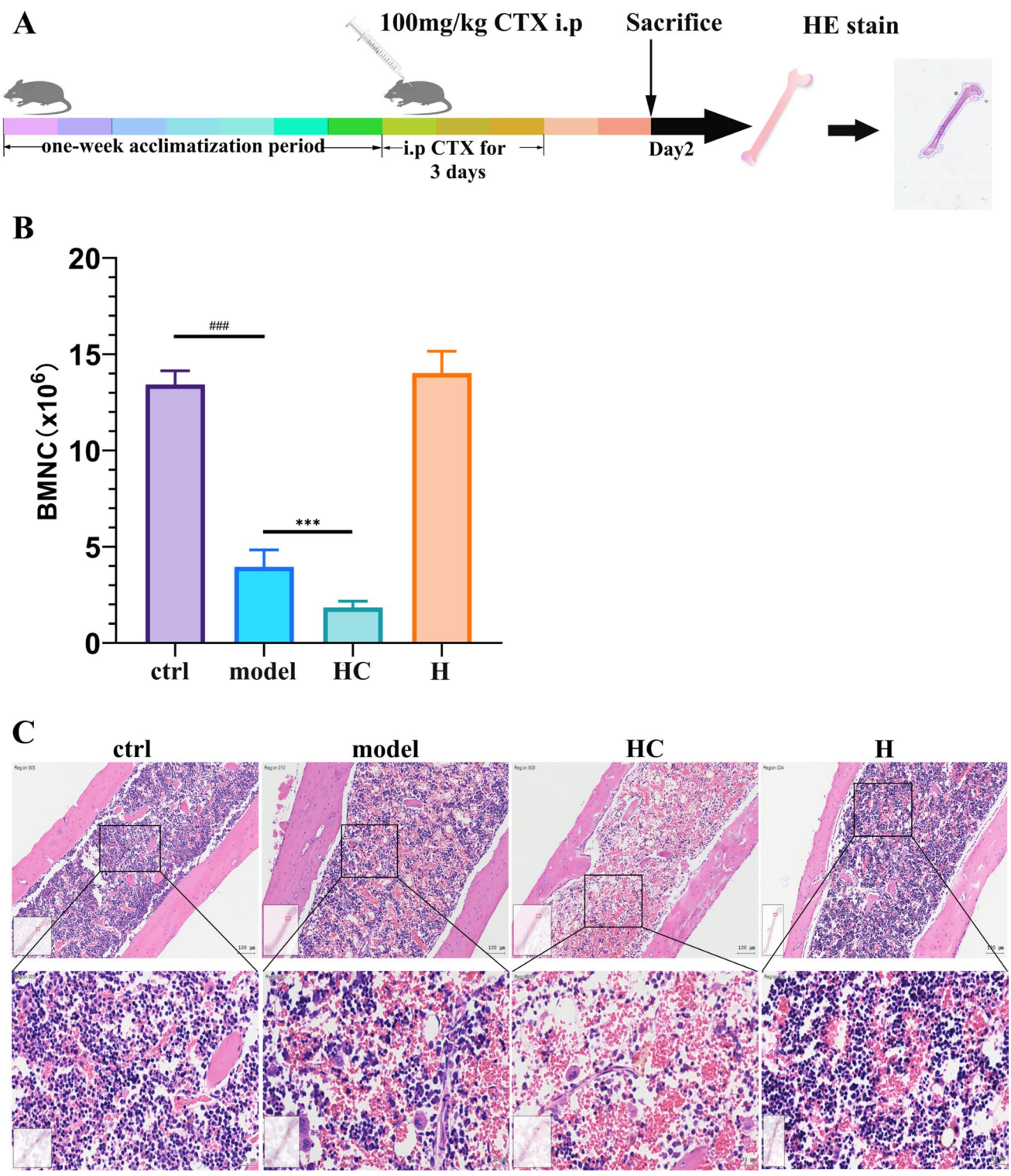


Fig. 2 Effects of high-altitude hypoxia on hematopoietic function in CTX-induced myelosuppression mice. **A** Experimental design. Refer to the "Materials and Methods" section for specific administration protocols. **B** The number of bone marrow nucleated cells (BMNCs) was counted on day 2 post-CTX treatment (n = 6). Results are expressed as mean ± SD. $^{###}P < 0.0001$ compared to the ctrl group; $^{***}P < 0.0001$ compared to the model group. **C** Representative H&E-stained sections of bone marrow are displayed for the indicated groups of mice. The scale bars in the upper and lower panels represent 100 μm and 20 μm, respectively

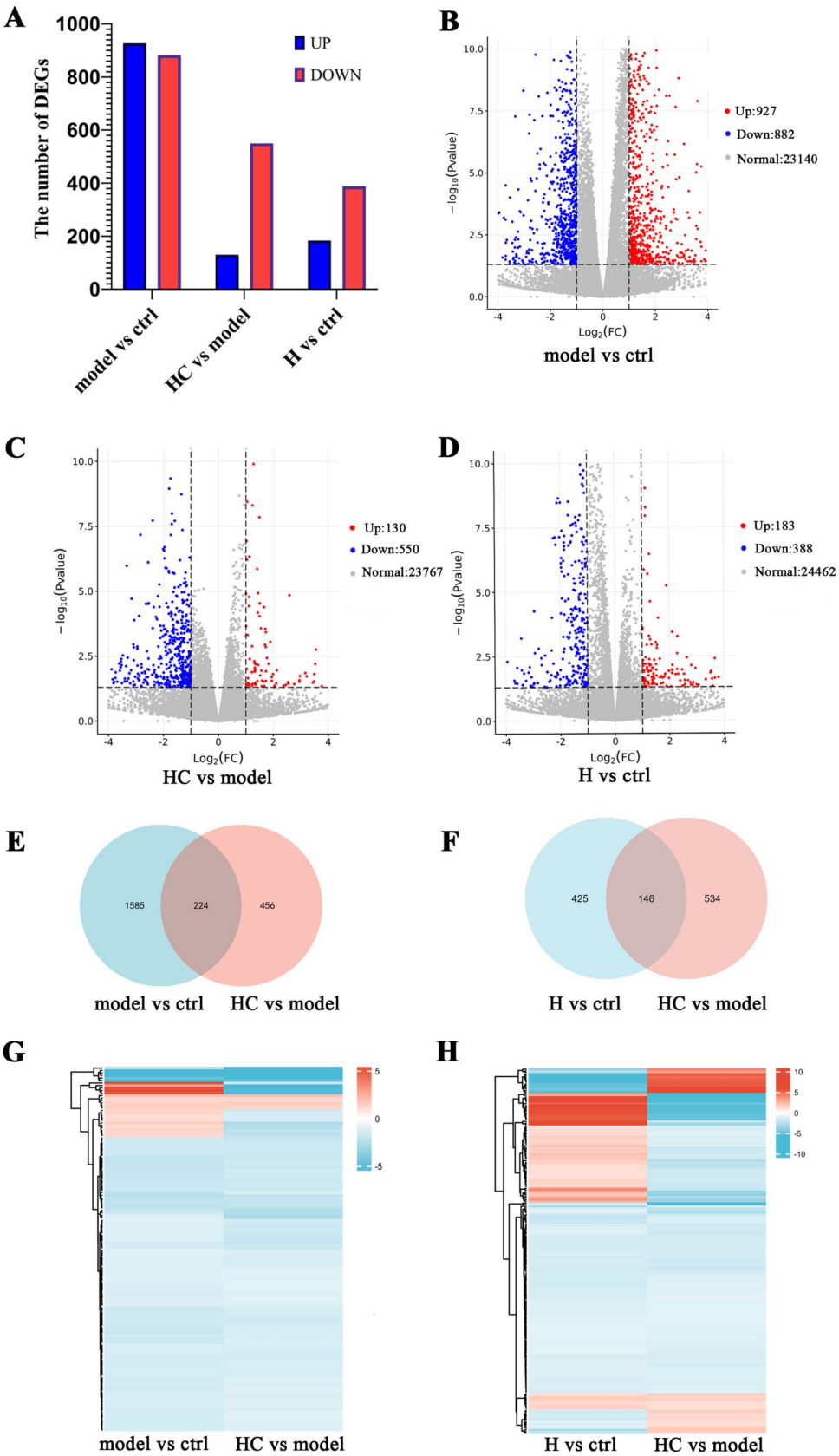


Fig. 3 Transcriptomic analysis. **A** Comparison of the number of upregulated and down-regulated genes among three groups: model vs ctrl, HC vs model, and H vs ctrl. **B**–**D** Volcano plots of differentially expressed genes (DEGs) for the three comparisons: model vs ctrl, HC vs model, and H vs ctrl. **E**, **F** Pie charts illustrating the co-regulated DEGs. **G**, **H** Heatmap of hierarchical clustering

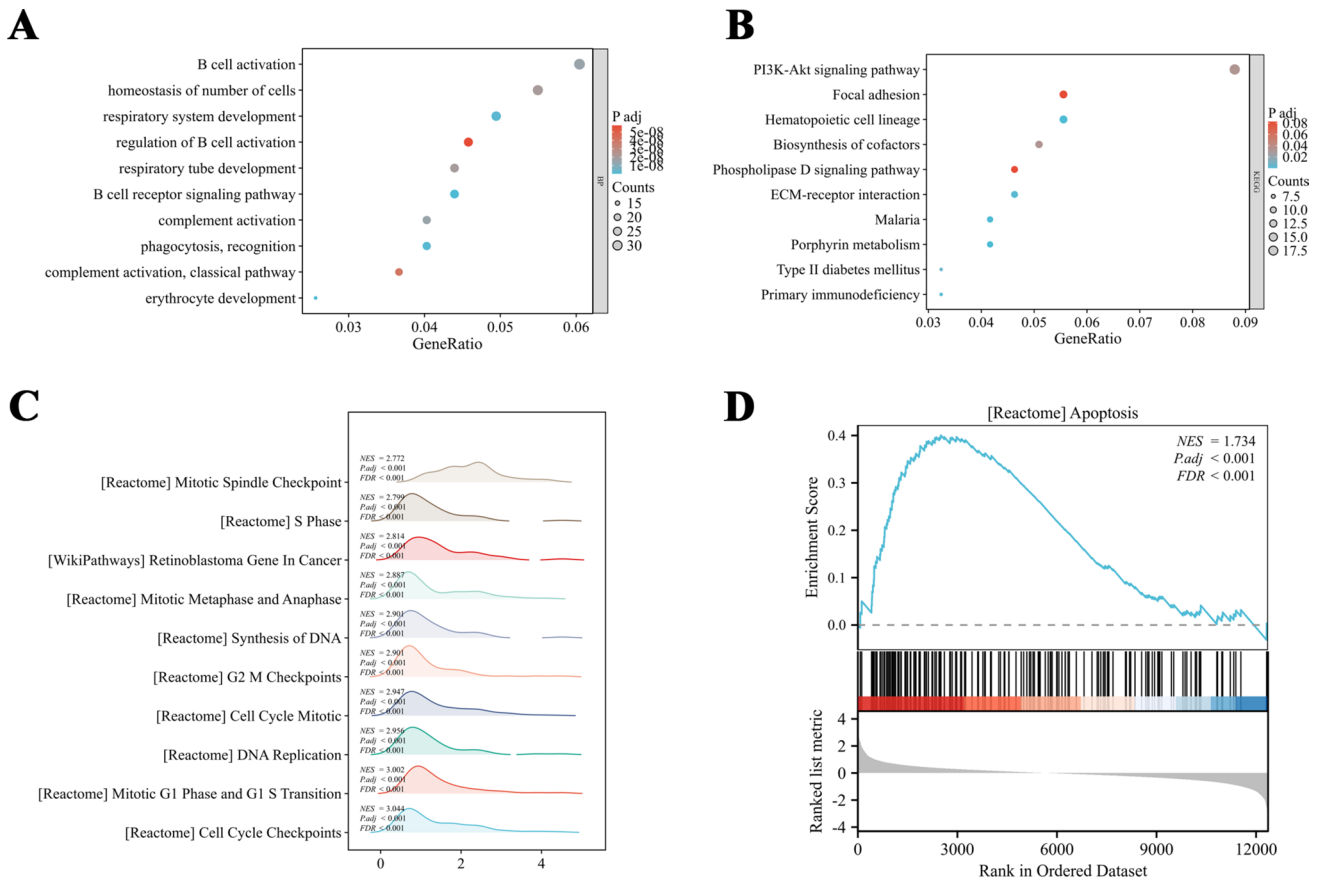


Fig. 4 Enrichment analysis. **A** GO analysis related to biological processes of DEGs. **B** KEGG enrichment analysis of DEGs. **C** Top 10 pathways enriched in Gene Set Enrichment Analysis (GSEA). **D** Apoptosis pathway enriched in GSEA

reported that cyclophosphamide can lead to a marked decrease in the number of white blood cells in peripheral blood, particularly reducing the counts of T cells and B cells, thereby diminishing the body's immune response capacity [24]. In this study, the pronounced decrease in WBC, granulocyte, lymphocyte, RBC, and BMNCs observed after CTX administration alone in the model group is a direct consequence of its mechanism of action. CTX induces DNA damage, cell cycle arrest, and apoptosis in hematopoietic stem and progenitor cells, leading to the suppression of erythropoiesis, granulopoiesis, lymphopoiesis, and overall hematopoiesis. This mechanistic explanation aligns with the observed data. In this study, the pronounced decrease in WBC, granulocyte, lymphocyte, RBC, and BMNCs observed after CTX administration alone in the model group is a direct consequence of its mechanism of action. CTX induces DNA damage, leading to cell cycle arrest at the G2/M checkpoint and activation of apoptosis in hematopoietic stem and progenitor cells. This results in the suppression of erythropoiesis, granulopoiesis, lymphopoiesis, and overall hematopoiesis. The observed increase in the proportion of cells in the S and G2/M phases, along with a decreased G0/G1 phase proportion, further supports the notion that CTX triggers DNA damage repair attempts and cell cycle arrest. These findings are consistent with the known mechanisms of CTX-induced myelosuppression and provide a clear explanation for the observed changes in peripheral blood and bone marrow cell counts.

Chemotherapy-induced myelosuppression is a significant clinical challenge, characterized by the suppression of bone marrow activity, leading to reduced production of blood cells [25, 26]. This condition severely impacts patients' immune function, increasing the risk of infections, anemia, and overall morbidity [27, 28]. The management of myelosuppression is crucial for the continuation of cancer therapy, as severe cases may need dose reductions, treatment delays, or discontinuation, which can adversely affect overall treatment outcomes [28, 29]. Our findings indicate a significant increase in the incidence of grade 3 or higher neutropenia among chemotherapy patients treated at high altitudes compared to those at low altitudes. This suggests that hypoxic conditions prevalent at higher altitudes may exacerbate the myelosuppressive effects of chemotherapy. Previous studies have primarily focused on the general effects of altitude on human physiology or the impact of chemotherapy on cancer patients, but none have specifically

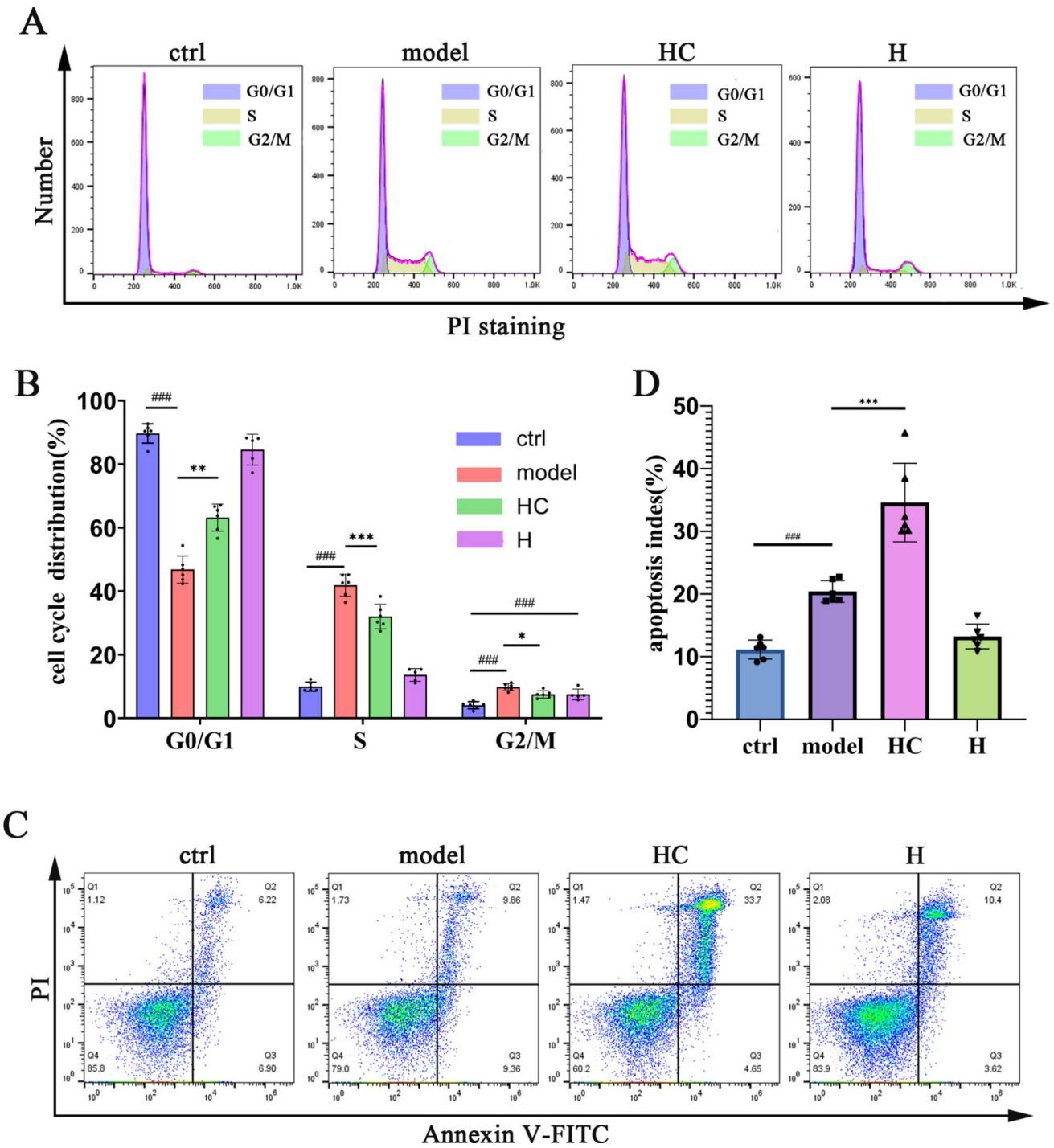


Fig. 5 Effect of hypoxia on the cell cycle and apoptosis of bone marrow nucleated cells (BMNCs) in CTX-induced myelosuppressed mice. **A** Propidium iodide (PI) staining was performed to assess the cell cycle of BMNCs using flow cytometric analysis. **B** Quantitative analysis of the durations of cell cycle phases (n = 6). **C** Flow cytometric analysis of apoptosis in BMNCs. **D** Quantitative analysis of apoptosis (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to the ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to the model group

examined the interaction between altitude and chemotherapy-induced myelosuppression in cancer patients. Thus, our study fills an important gap in the literature by highlighting the potential risks associated with administering chemotherapy at high altitudes and underscores the need for tailored treatment strategies to mitigate these risks. This study's findings are particularly relevant for healthcare providers in high-altitude regions, as they may need to

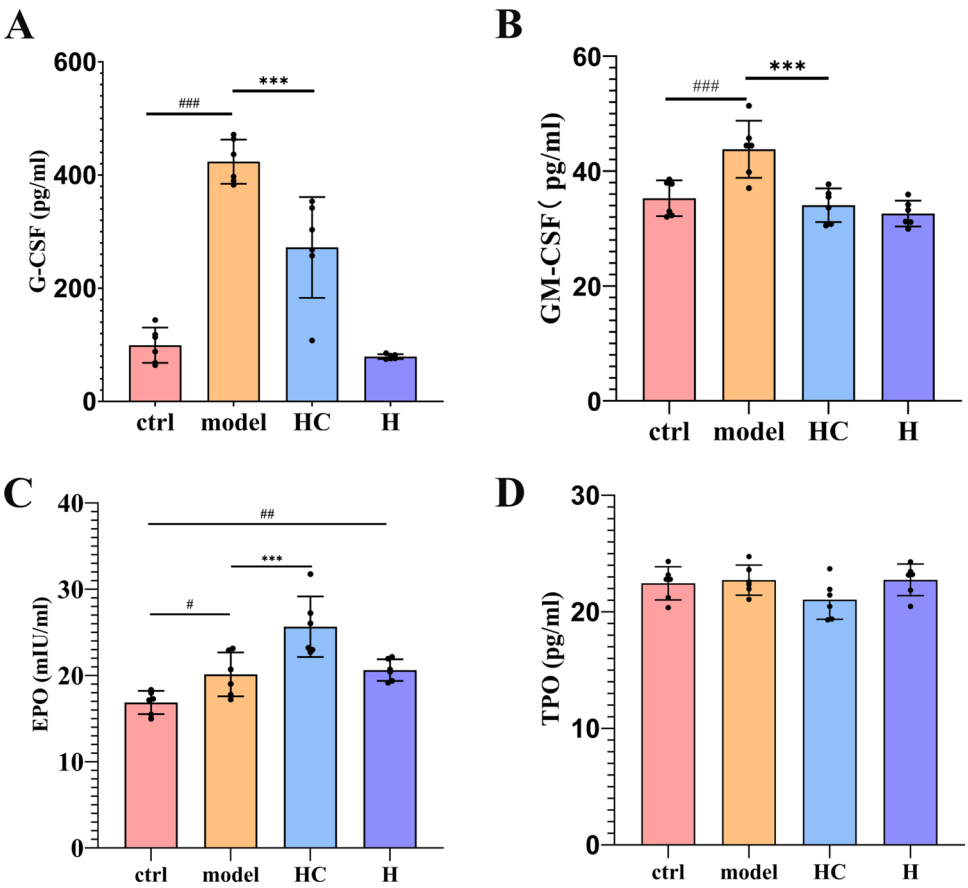


Fig. 6 The levels of G-CSF (**A**), GM-CSF (**B**), EPO (**C**), and TPO (**D**) in serum were detected on day 2 after chemotherapy. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to the ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to the model group

adopt more rigorous monitoring and supportive care protocols for patients with breast cancer undergoing chemotherapy. Large randomized controlled studies are needed for further confirmation.

In patients with breast cancer, hormone receptor (HR)-positive breast cancer accounts for approximately 70%-80% of all breast cancer cases [30]. Hormone receptor-negative breast cancers are typically more aggressive and have fewer treatment options compared to hormone receptor-positive cancers [31]. Endocrine therapy, a primary treatment strategy for HR-positive patients, significantly improves disease-free survival and overall survival [31, 32]. The molecular characteristics of breast cancer, such as ER status, may influence the occurrence and severity of neutropenia [33]. In our study, we observed that the plateau group had a significantly higher incidence of grade 3 or higher neutropenia and overall neutropenia compared to the plain group. Interestingly, the plateau group also exhibited a relatively higher proportion of HR-positive patients compared to the plain group, although this difference did not reach statistical significance. This observation suggests that HR status may play a role in modulating the severity of chemotherapy-induced myelosuppression, particularly in high-altitude environments. Estrogen has been shown to play a protective role in bone marrow function by promoting the proliferation and differentiation of hematopoietic stem cells through its receptors [34, 35]. In HR-positive patients, the presence of estrogen may help mitigate the severity of chemotherapy-induced myelosuppression. Conversely, estrogen deficiency, which is more likely in HR-negative patients, can lead to a decline in hematopoietic function, increasing the risk of myelosuppression. The high incidence of neutropenia in the plateau group, despite the higher proportion of HR-positive patients, may be attributed to the combined effects of high-altitude hypoxia and chemotherapy. This suggests that HR status may play an important role in aggravating the severity of chemotherapy-induced myelosuppression in high-altitude environments. These findings highlight the potential impact of HR status on patient treatment and prognosis, particularly in the context of high-altitude hypoxia. However, further validation through prospective, multicenter, large-sample studies is necessary to confirm these observations and to explore the underlying mechanisms.

Understanding the mechanisms underlying myelosuppression is crucial for developing effective therapeutic strategies to mitigate these adverse effects and improve patient outcomes. Despite extensive research on the regulation of erythropoiesis, the precise effects of hypoxia on WBC remain indeterminate [12, 36–38]. To the best of

our knowledge, this study is the first to investigate the impact of high-altitude hypoxia on hematopoietic function following chemotherapy, along with the underlying mechanisms involved. The present study specifically investigates the impact of high-altitude hypoxia on chemotherapy-induced myelosuppression in mice, aiming to elucidate the multifaceted effects of hypoxic conditions on bone marrow function and hematopoiesis. According to a recent study, hypobaric hypoxia enhances the proliferation and apoptosis of bone marrow erythroblasts in rats, leading to increased peripheral blood red blood cell parameters [39]. Consistently, our research demonstrates that high-altitude hypoxia significantly increases apoptosis of BMNCs in mice. Further, the present study demonstrated that high-altitude hypoxia significantly increased proportion of cells in the G0/G1 phase and reduced proliferation index in the CTX-induced myelosuppressive mice. These findings provide a deeper understanding of how hypoxia modulates cellular responses to chemotherapy, revealing that hypoxia not only impairs cell proliferation but also promotes apoptosis.

Understanding the mechanisms underlying myelosuppression is crucial for developing effective therapeutic strategies to mitigate these adverse effects. Previous studies have made some progress in understanding how hypoxia conditions affect bone marrow cell proliferation and differentiation [39, 40]. However, these studies usually focus on a single factor and lack a systematic and comprehensive analysis. The findings of our study indicate that high-altitude hypoxia exacerbates myelosuppression in mice undergoing chemotherapy, particularly through its effects on serum hematopoietic cytokines. The elevated EPO levels in hypoxic conditions reflect that hypoxia can stimulate erythropoiesis that is crucial for maintaining Hb levels and enhancing RBC production, which is consistent with many previous studies [41, 42]. From this side, high-altitude hypoxia seems as a good factor for patients undergoing chemotherapy. For cancer patient living in high-altitude environment, the increased levels of EPO and RBCs could indeed indicate enhanced oxygen transport in the blood, which might theoretically make tumors more susceptible to radiotherapy due to improved re-oxygenation. This is an intriguing hypothesis that could have significant clinical implications for personalized radiotherapy strategies, thereby pointing out a new direction for our future research. However, the significant reduction in G-CSF and GM-CSF levels observed in the HC group suggests that there is a relation between high-altitude hypoxia and the decrease in WBC and granulocyte levels. It is well known that G-CSF and GM-CSF play a multifaceted role in regulating hematopoiesis, which are crucial for the production of granulocyte [43, 44]. WBC mainly include granulocyte and lymphocyte [45, 46], therefore, the depletion of G-CSF and GM-CSF will induce the decline in WBC and granulocyte. These cytokines are essential for maintaining hematopoietic homeostasis, and the decline induced by hypoxia highlights the challenges faced by patients undergoing chemotherapy at high altitudes. Future studies should explore potential interventions that could mitigate these adverse effects, aiming to improve the management of chemotherapy-induced myelosuppression in hypoxic environments.

PI3K-Akt pathway is an important biological pathway in cell signal transduction, which is involved in various physiological processes such as cell growth, proliferation, and apoptosis [47, 48]. Studies have shown that PI3K-Akt signaling pathway plays a key role in the proliferation and survival of hematopoietic stem cells (HSCs) and are crucial for maintaining hematopoietic stem cell (HSC) function and bone marrow microenvironment integrity [49, 50]. In acute lymphoblastic leukemia cells, inhibition of PI3K-Akt pathway leads to decreased expression of Bcl-2, thus promoting apoptosis [51]. Therefore, high-altitude hypoxia is likely to regulate the proliferation and apoptosis of bone marrow cells through the PI3K-Akt pathway, and further aggravate myelosuppression after chemotherapy. This mechanistic understanding highlights the potential for targeting PI3K-Akt pathway to mitigate the adverse effects of hypoxia on bone marrow function during chemotherapy, thereby improving patients' outcomes.

Despite the known half-life of cyclophosphamide of up to 12 h, the blood biochemical values in mice remained abnormal 21 days after a three-day treatment regimen, suggesting a complex and persistent impact on hemopoietic systems. The protracted recovery may be due to the cumulative toxic effects of cyclophosphamide on the bone marrow and other rapidly dividing cells, leading to prolonged hematopoietic suppression. Additionally, the drug's alkylating activity could result in sustained damage to the liver and kidneys, which are crucial for drug metabolism and excretion, respectively. The slow regeneration of these organs, combined with potential oxidative stress and inflammation, may further delay the return to baseline biochemical parameters. Moreover, the immune system's response to chemotherapy could contribute to the variability in recovery times of myelosuppression. These findings underscore the need for careful dosing, monitoring of drug metabolism, and the implementation of supportive care strategies to mitigate the cytotoxic effects of cyclophosphamide in future studies.

5 Conclusion

High-altitude hypoxia significantly exacerbates chemotherapy-induced myelosuppression, which may be related to reducing the serum level of G-CSF/GM-CSF and regulating apoptosis and proliferation by PI3K-Akt signaling pathway. These findings imply that cancer patients undergoing chemotherapy in high-altitude regions necessitate enhanced supportive care to mitigate these adverse effects.

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Author contributions JS contributed to acquisition and interpretation of data and drafting of the manuscript. FZ, TQ, DR, ZL, and JM contributed to technical support and participated in data extraction. JZ revised it critically for important intellectual content and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

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Availability of data and materials The RNA sequencing data generated in the present study may be found in the Sequence Read Archive (SRA) Database under the project accession number PRJNA1176344.

Declarations

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Affiliated Hospital of Qinghai University (approval number P-SL-2023-447).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication All authors agreed to publish.

Competing interest The authors declare no competing interests.

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