

ZhenQi FuZheng Formula Improves Mouse Hematopoietic Function after Cyclophosphamide-Induced Damage via Enhancing Macrophage Colony-Stimulating Factor Concentrations

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Research

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Abstract

Background: Among the hematopoietic system, bone marrow has been recognized as the major source of hematopoietic progenitor cells (HPCs). Immunosuppression can inhibit the growth of bone marrow cells and cyclophosphamide (CTX) has been reported to induce myelosuppression. Regeneration of the immune system and hematopoietic function has become a primary problem in tumor adjuvant therapy. The present study investigated the effects of ZhenQi FuZheng formula (ZQFZ) on hematopoietic function in cells and murine models of cyclophosphamide(CTX)-induced immunosuppression and hematopoietic dysfunction.

Methods: According to antibody chip analysis and enzyme-linked immunosorbent assay, ZQFZ regulated seven cytokines in serum and the spleen.

Results: In Nrf2-siRNA transfected K562 cells, the regulatory effects of ZQFZ on the expression concentrations of macrophage colony-stimulating factor (M-CSF) and hematopoietic related proteins were strongly abolished. In immunosuppressed mice, ZQFZ enhanced the NK cell activities and regulated the concentrations of cytokines related to immune function. ZQFZ induced proliferation and differentiation, and upregulated the expression of hematopoietic-related proteins, including p90 ribosomal S6 kinases 1 (RSK1p90), c-Myc, and ETS transcription factor in CHRF and/or K562 cells. In mice with hematopoietic dysfunction, ZQFZ contributed to the recovery of blood cell concentrations in peripheral blood to normal values and enhanced the percentages of B lymphocytes (LYs) and juvenile cells in the bone marrow.

Conclusions: In spleens of hematopoiesis damaged mice and primary cultured bone marrow cells, ZQFZ upregulated the expression concentrations of NF-E2-related Factor 2 (Nrf2) and its downstream proteins, and the activation of nuclear factor kappa-B. ZQFZ showed remarkably beneficial effects on the bone marrow hematopoietic system, at least partially, by enhancing the concentrations of M-CSF.

1 Background

Among the hematopoietic system, bone marrow has been recognized as the major source of hematopoietic progenitor cells (HPCs), while the spleen stores blood cells and removes harmful substances such as abnormal red blood cells, and other substances in the blood[1, 2]. Except for the hematopoietic microenvironment, the hematopoietic regulatory factors, such as interleukin (ILs), colony stimulating factors (CSFs), and chemokines, help to maintain the function of hematopoiesis[3]. Hematopoietic dysfunctions, such as immunosuppression, hematopoietic inhibition, and myelosuppression, have been noted in patients with cancer who undergo long-term radiotherapy and chemotherapy[4, 5]. Immunosuppression can inhibit the growth of bone marrow cells[6]. Cyclophosphamide (CTX), a widely used constituent of combination chemotherapy regimens, has been reported to induce myelosuppression[7]. Regeneration of the immune system and hematopoietic function has become a primary problem in tumor adjuvant therapy[7].

The hyper-levels of reactive oxygen species (ROS), one of the phenomena and pathological mechanisms that occurs during oxidative stress, is responsible for hematopoietic dysfunction by causing bone marrow failure and hematopoietic malignancies[8]. Increased concentrations of ROS during oxidative stress are toxic to hematopoietic stem/progenitor cells and persturbs the differentiation of hematopoietic cells, and it affects the life span of erythrocytes in mature blood cells[8, 9]. Activated nuclear factor-E2-related factor 2 (Nrf2) leads to the suppression on inflammatory factors[10], and promotes the proliferation and differentiation of hematopoietic stem cells (HSCs)

by affecting the long-term hematopoietic cell cycle[11]. Theaflavins protect HSCs from hematopoietic damage caused by ionizing radiation by reducing ROS concentrations by activating Nrf2[12].

In the clinic, the injection of recombinant human granulocyte colony-stimulating factor (rhG-CSF) is commonly used to treat myelosuppression and can prevent chemotherapy-induced neutropenia and enhance the function of mature neutrophils[13]. However, adverse effects of rhG-CSF have been noted, such as the proliferation of cancer cells[14]. In contrast, traditional Chinese medicine has been reported to have protective effects on bone marrow[15]. A combination of *Astragalus membranaceus* and *Angelica sinensis* stimulate hematopoietic function, as evidenced by its promotion of the recovery of peripheral blood cells and bone marrow nucleated cells (BMNCs), and its acceleration of the proliferation of HSCs and HPCs in mice with hematopoietic dysfunction[16, 17]. ZhenQi FuZheng formula (ZQFZ), a compound prescription of astragali radix (*Astragalus propinquus* Schischkin) and glossy privet fruit (*Ligustrum lucidum* W.T.Aiton), was firstly put forward on 1974 in China by Professor Yan Sun, and has been used to enhance the immune function and protect against bone marrow and adrenal damage caused by various other diseases, surgery, radiation, and/or chemotherapy in clinics (drug approval number Z20053398). Astragali radix and glossy privet fruit are recorded as top grade in The Herbal Classic Shen Nong, and both possess immunomodulatory, anti-inflammatory and anti-oxidant effects[18, 19]. Based on the previous results, we speculate that ZQFZ improves hematopoietic function, especially hematopoietic suppression induced by chemotherapeutic drugs.

In this experiment, we found that ZQFZ promoted the differentiation of hematopoietic cells, enhanced immune function in immunosuppressed mice, and protected the hematopoietic system in hematopoietic damaged mice. These effects are related to the effects of ZQFZ on the concentration of macrophage colony stimulating factor (M-CSF), which may be modulated via Nrf2 and nuclear factor-kappa B (NF-κB) signaling.

2 Methods

2.1 The detection of effective components of ZQFZ via HPLC

The effective components of ZQFZ according to Chinese Pharmacopoeia 2015 version were realization via high performance liquid chromatography (HPLC) method by Shimadzu chromatograph equipped with C18 reverse phase column (250 mm × 4.6 mm, 5 μm) (E2912829, Liaoning, China) under the ultraviolet detector at the wavelength of 224 nm. The conditions of HPLC as follows. The column temperature was 30 °C, the injection volume was 10 μL, the mobile phase for 0–5, 5–20, 20–35, 35–50, 50–65 and 65–80 min were 100%-95%, 95% – 85%, 85% – 75%, 75% – 65%, 65%-55% and 55% – 50% of acetonitrile dissolved in ultrapure water and the flow rate was 1.0 mL/min.

2.2 Cell culture

K562 (CCL-243™) (human myelogenous leukemia) and CHRF (CRL10107) (human megakaryoblastic leukemia) cell lines (American Type Culture Collection, ATCC, USA), were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Invitrogen, California, USA) with 15% fetal bovine serum (FBS) (Invitrogen Life Technologies, Karlsruhe, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies, Karlsruhe, Germany) in a humidified incubator at 37 °C with 5% CO₂ and 95% air overnight. The viability of the isolated cells was > 95%.

From the femurs and tibias of 6–8 week male BALB/c mice, the bone marrow cells were flushed out and collected with Dulbecco's Modified Eagle Medium (DMEM) (HyClone, South Logan, UT, USA) using a 1 mL syringe with a 21-gauge needle. Bone marrow mononuclear cells were collected with the red blood cell lysis buffer (GS3309) (Genview, Pompano Beach, FL, USA) by removing non-nucleated cells. The resultant cells were washed and pelleted with DMEM, seeded into a 6-well plate (2×10^6 cells/well) in DMEM containing with 10% FBS, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 100 U/mL penicillin, and cultured at 37 °C in a humidified incubator with 5% CO₂ and 95% air.

2.3 XTT and apoptosis assay

The seeded CHRF or K562 cells were incubated with 0, 25, or 100 $\mu\text{g}/\text{mL}$ ZQFZ for 24 h, and the Cellular proliferation was detected by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) method as described previously[20].

According to previous research[21], the seeded CHRF or K562 cells were incubated with 0, 25, and 100 $\mu\text{g}/\text{mL}$ ZQFZ for 24 h, exposed to Annexin V & Dead Cell Reagent (4700 – 1485) for another 20 min at 25 °C in darkness, and finally analyzed using the Muse™ Cell Analyzer (EMD Millipore, Billerica, MA, USA).

2.4 Transfection of Nrf2-siRNA

The seeded K562 cells (2×10^5 cells/well) were transfected via incubating with 125 μL mixtures containing 125 μL of 1640 medium, 100 pmol of Nrf2-siRNA (R10043.8) (RiboBio, China) with sequence of 5'-GGATGAAGAGACCGGAGAA-3' and 4 μL of LipoRNAi™ Transfection Reagent (Beyotime), for 48 h according to the manufacturer's protocol. The Nrf2-siRNA transfected K652 cells were incubated with 0 and 100 $\mu\text{g}/\text{mL}$ ZQFZ for 24 h. The cells were then harvested for western blotting.

2.5 Erythroid differentiation of K562 cells

Seeded K562 cells (4×10^5 cells/well) were incubated 0, 25, and 100 $\mu\text{g}/\text{mL}$ of ZQFZ for 24 h. The erythroid differentiation and hemoglobin (HGB) of K562 cells were detected using Benzidine staining the same as our previous study[20].

2.6 Evaluation in CTX-injected mice with immunosuppression

The experimental protocol (2017SY0603) has been approved by the Institution Animal Ethics Committee of Jilin University. Seventy 7-week male BALB/c mice weighted 18–22 g (specific pathogen-free (SPF) grade, SCXK(Liao)-2015-0001) were housed in a controlled environment the same as our previous study[20].

After one-week acclimatization, fifty mice were intraperitoneally injected (i.p.) with 75 mg/kg of CTX (AMRESCO, Boise, Idaho, USA) dissolved in normal saline (NS) for 3 days, and then randomly divided into five groups, gavage with 10 mL/kg of double distilled (D.D.) water serving as model group (n = 10), 4.5 mg/kg of transfer factor oral liquid (TFO) (dissolved in D.D. water) (n = 10), 0.1 g/kg (n = 10), 0.3 g/kg (n = 10) and 0.9 g/kg (n = 10) of ZQFZ (dissolved in D.D. water) once per day for 4 weeks. CTX (60 mg/kg) was injected once per week for maintaining immunosuppression in mice. Another twenty mice were injected (i.p.) with NS for 3 days, and then gavaged with 10 mL/kg of D.D. water serving as the control group (n = 10), and 0.3 g/kg of ZQFZ serving as ZQFZ monotherapy group (n = 10) once per day for 4 weeks. All mice were monitored every day during the whole experiment. 2-h after the last administered, blood was collected from the caudal veins, and then mice were euthanasia by injecting 200 mg/kg of pentobarbital.

2.6.1 Natural killer (NK) cell cytotoxic activity assay

The NK cell cytotoxic activity detection was performed the same as our previous study[22]. Spleen cells of each mouse were obtained by filtering the fresh spleens through the 200 mesh screen. After collection and resuspension, 100 μ L of spleen cells (5×10^7 cells/mL) and 100 μ L of YAC-1 cells (1×10^6 cells/mL) (TIB-160TM) (ATCC, Manassas, VA, USA) were suspended with RPMI1640 medium containing 10% FBS were seeded into 96-well plates, and cultured at 37 °C in a 5% CO₂ incubator for 4 h. 1% NP-40 treated cells was served as maximum control. The commercial kit (ml002267), obtained from Enzyme-linked Biotechnology (Shanghai, China) was applied to detect the concentration of lactate dehydrogenase (LDH) in 100 μ L culture medium. According to the absorbance and the following equation, the NK cell cytotoxic activity was calculated using the same formula as our previous study[22].

2.6.2 Histopathological analysis

Similar as our previous study[20], the pathological alterations of spleen of mice with immunosuppression were detected using the hematoxylin-eosin (H&E) staining under the inverted microscope CKX41 (Olympus, Tokyo, Japan).

2.6.3 Cytokines detection

The levels of immunoglobulin (Ig)A (KT2055-A), IgG (KT2057-A), IgM (KT2058-A), interleukin-1 β (IL-1 β) (KT2040-A), -2 (KT2795-A), -6 (KT2163-A) and -10 (KT2176-A), tumor necrosis factor (TNF)- β (KT2131-A), interferon (IFN)- α (KT2366-A) in serum of mice with immunosuppression were detected using the ELISA via the related commercial kits obtained from Jiangsu Kete Biological Technology Co., Ltd., Jiangsu, China, according the manual structures.

2.7 The experimental performed in CTX-injected mice with hematopoietic dysfunction

The experimental protocol (2017SY0603) has been approved by the Institution Animal Ethics Committee of Jilin University. Seventy 7-week male BALB/c mice weighted 18–22 g (SPF grade, SCXK(Ji)-2016-0008) were housed in a controlled environment the same as our previous study[20].

After one-week acclimatization, fifty mice were injected (i.p.) with 100 mg/kg of CTX dissolved in NS for three days, and then randomly divided into five groups, gavage with 10 mL/kg of D.D. water serving as model group (n = 10), and 0.1 g/kg (n = 10), 0.3 g/kg (n = 10) and 0.9 g/kg (n = 10) of ZQFZ (dissolved in D.D. water) once per day for 4 weeks, and subcutaneously injecting with 22.5 μ g/kg of rhG-CSF (n = 10) (Nanjing Yixun Biotechnology Co., Ltd., Nanjing, China) twice a week for 4 weeks. CTX at dose of 80 mg/kg was injected every Monday to avoid the restoration of hematopoietic function. The control group (n = 10) and ZQFZ monotherapy group (n = 10) were obtained similarly as 2.6. All mice were monitored every day during the whole experiment.

2.7.1 Detection of biochemical indexes in peripheral blood

Blood was sampled 2-h after the last treatment from the caudal veins for peripheral blood analysis immediately with a fully automatic blood analyzer (Drew Scientific Group, Dallas, TX, USA).

2.7.2 BMMNCs component analysis

200 mg/kg of pentobarbital injection (i.p.) was used for euthanasia to mice. Under the sterile condition, the tibial and femoral section were collected immediately. Similar to our previous research, the ACK lysis buffer was used

for the isolation of the bone marrow mononuclear cells (BMMNCs)[20]. The antibodies related to surface markers including FITC-conjugated anti-mouse Lineage Cocktail (133302), APC-conjugated anti-mouse CD19 (152410), and PerCP-conjugated anti-mouse CD45 (103129) were applied to stain with cells at 25 °C for 15 min darkly. APC-conjugated anti-rat IgG2b (400611), PerCP-conjugated anti-rat IgG2b (400629), FITC-conjugated anti-rat IgG2b (400605) and FITC-conjugated anti-rat IgG2a (400505) were set as isotype controls. The experimental antibodies were purchased from Biolegend (San Diego, CA, USA). The Cytoflex flow cytometer (Beckman Coulter, California, USA) was applied for analyzing.

2.7.3 Histopathological analysis

Similarly, the pathological alterations of femoral condyle, liver, spleen and kidney of mice with immunosuppression were detected using the H&E staining[20].

2.7.4 High-throughput antibody chip analysis

The 40 cytokines/chemokines of the spleen in hematopoiesis damaged mice were analyzed using the Mouse Cytokine Array Panel A Kit (R&D Systems, Minneapolis, MN, USA) the same as our previous research[20].

2.7.5 Cytokines/chemokines detection

Cytokines/chemokines including IL-2 (KT2795-A), IL-5 (KT2164-A), macrophage colony stimulating factor (M-CSF) (KT2156-A), TNF- α (KT2132-A), monocyte chemoattractant protein (MCP)-1 (KT2082-A), MCP-5 (KT9380-A), macrophage inflammatory protein 1 α (MIP-1 α) (KT2154-A) and ROS (KT2800-A) in spleen and/or serum of hematopoiesis damaged mice were measured using ELISA kits (Jiangsu Kete Biological Technology Co., Ltd.).

2.7.6 Western blotting

The primary cultured bone marrow cells, the Nrf2-siRNA transfected K652 cells, the CHRF and K562 cells were treated with ZQFZ at doses of 0, 25 and/or 100 μ g/mL for 24 h. The treated cells and the spleens of hematopoiesis damaged mice were lysed using the radioimmunoprecipitation assay lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich) and 2% phenylmethanesulfonyl fluoride (Sigma-Aldrich). 30–40 μ g of proteins were separated using 10%-12% SDS-PAGE and transferred onto a polyvinylidene difluoride membranes (0.45 μ m, Merck Millipore, Billerica, MA, USA). After blocking with 5% bovine serum albumin (BSA), the membranes were incubated with primary antibodies as Table S1 at 4 °C overnight, following with the exposure to horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted to 1:2000) (NBP2-30347H and NBP2-30348H) (Novus Biologicals, Littleton, Colorado, USA) for 4 h at 4 °C. An enhanced chemiluminescence detection kit (Merck Millipore) combining with an imaging system (BioSpectrum600) were applied to visualize the protein bands. The ImageJ software (Version 1.8.0) (National Institutes of Health, Bethesda, MD) was used to analyze the pixel density of the band.

2.8 Statistical analysis

A one-way ANOVA followed by a Tukey's post hoc test comparison was applied for statistical significance analysis using SPSS 16.0 software (IBM Corporation, Armonk, NY, USA). Data are expressed as mean \pm S.D. and considered significant at $p < 0.05$.

3 Results

3.1 Detection of effective components of ZQFZ

Based on the 2015 version of the Chinese Pharmacopoeia, the specific composition of ZQFZ was determined by HPLC. Thus, ZQFZ was found to contain 3.934 mg/g of salidroside, 0.138 mg/g of calycosin-7-glucoside, 0.166 mg/g of ligustroflavone, 0.026 mg/g of ononin, 0.076 mg/g of quercetin, and 0.017 mg/g formononetin (Fig.S1a-f).

3.2 Immunomodulatory activity of ZQFZ in mice with CTX-induced immunosuppression

NK cells exhibit specific immunomodulatory effects by identifying ligands of target cells and then directly killing these cells[23]. In the spleens of mice with CTX-induced immunosuppression, similar to TFO, ZQFZ enhanced the cytotoxic activities of NK cells by more than 60.4% ($p < 0.001$) (Fig.S2a). CTX-injection caused narrowed white pulp, enlarged red pulp and an unclear boundary between the white and red pulp in the spleen of mice with CTX-induced immunosuppression; in contrast, ZQFZ repaired these pathological changes (Fig.S2b).

In the immune system, immunoglobulins can identify and neutralize exogenous pathogenesis, while ILs can regulate the proliferation of immune cells[24]. Compared with vehicle-treated immunosuppressed mice, ZQFZ dosed at 0.9 g/kg resulted in 12.4% ($p < 0.05$), 21.5% ($p < 0.01$), and 11.8% ($p < 0.05$) enhancement of IgA, IgG, and IgM concentrations in sera (Table 1), respectively. Moreover, ZQFZ dosed at 0.9 g/kg resulted in a 19.4% reduction of IL-1 β ($p < 0.001$) and increases of 29.2%, 9.8%, and 9.7% in IL-2 ($p < 0.001$), IL-6 ($p < 0.05$), and IL-10 ($p < 0.01$) concentrations in the sera of mice with CTX-induced immunosuppression (Table 1). IFN is involved in the immunostimulatory and immunomodulatory processes[25]. ZQFZ dosed at 0.9 g/kg increased the serum concentrations of IFN- α ($p < 0.01$) and at 0.1 g/kg enhanced the serum concentrations of TNF- β ($p < 0.05$) in mice with CTX-induced immunosuppression (Table 1). Compared with the effects in healthy mice, ZQFZ alone showed no significant effects on these immune-related factors (Table 1).

Table 1
The effects of ZQFZ on serum cytokines of CTX-injected mice with immunosuppression

	CTRL	CTX 75 (mg/kg)					
		-	TFO	ZQFZ (g/kg)			
			(mg/kg)	0.1	0.3	0.9	0.3
		4.5	0.1	0.3	0.9	0.3	
IgA (µg/ml)	92.0 ± 8.6	83.9 ± 8.0 [#]	87.6 ± 9.0	87.5 ± 6.2	88.8 ± 7.9	94.3 ± 7.8 [*]	90.4 ± 15.9
IgG (mg/ml)	7.2 ± 0.7	6.1 ± 0.9 ^{##}	6.4 ± 0.9	7.0 ± 0.8	7.4 ± 0.9 ^{**}	7.4 ± 0.8 ^{**}	7.4 ± 0.6
IgM (µg/ml)	1005.4 ± 70.8	863.7 ± 38.4 ^{###}	954.4 ± 67.3 ^{**}	843.8 ± 65.0	861.9 ± 61.2	965.6 ± 80.8 [*]	935.6 ± 64.6
IL-1β (pg/ml)	31.2 ± 3.0	34.0 ± 2.0 [#]	28.3 ± 4.1 ^{**}	32.8 ± 3.5	28.8 ± 6.2 [*]	27.4 ± 4.2 ^{***}	33.5 ± 1.3
IL-2 (pg/ml)	72.9 ± 7.1	64.5 ± 8.0 [#]	74.3 ± 6.2 ^{**}	81.7 ± 7.5 ^{***}	83.2 ± 7.2 ^{***}	83.3 ± 9.1 ^{***}	81.9 ± 10.4 [#]
IL-6 (pg/ml)	40.2 ± 3.7	33.3 ± 2.9 ^{###}	36.6 ± 3.8 [*]	37.3 ± 2.4 ^{**}	37.1 ± 3.5 [*]	36.6 ± 2.0 [*]	38.4 ± 1.6
IL-10 (pg/ml)	358.0 ± 15.9	335.2 ± 16.9 [#]	347.3 ± 17.6	341.6 ± 27.1	341.8 ± 26.2	367.7 ± 12.2 ^{**}	347.2 ± 12.6
TNF-β (pg/ml)	59.8 ± 3.9	63.8 ± 4.1 [#]	58.1 ± 5.3 [*]	57.8 ± 5.8 [*]	60.5 ± 7.7	63.5 ± 8.8	58.1 ± 15.6
IFN-α (pg/ml)	12.9 ± 1.0	11.1 ± 1.0 ^{###}	11.8 ± 0.8	11.7 ± 0.7	11.9 ± 1.3	13.2 ± 1.9 ^{**}	12.0 ± 1.9

Data are expressed as mean ± S.D. (n = 10/group) and analyzed using a one-way analysis of variance followed by Tukey's test. [#]*P* < 0.05, ^{##}*P* < 0.01 and ^{###}*P* < 0.001 vs. control group, ^{*}*P* < 0.05, ^{**}*P* < 0.01 and ^{***}*P* < 0.001 vs. model group. ZQFZ, ZhenQi FuZheng formula, CTX, cyclophosphamide, TFO, transfer factor oral liquid.

3.3 ZQFZ promotes proliferation and differentiation of hematopoietic cells

The CHRF and K562 cell lines are commonly used to study cell proliferation and differentiation related to hematopoietic function[26, 27]. Incubation of ZQFZ for 24 h, especially at 100 µg/mL, promoted the proliferation of CHRF (*p* < 0.01) and K562 cells (*p* < 0.001) (Fig. 1a) without influencing their apoptotic rate (Fig. 1c and d). ZQFZ at 100 µg/mL improved erythroid differentiation of K562 cells, as indicated by an increased benzidine positive cell rate *v* (*p* < 0.001) (Fig. 1b). The c-Myc, ETS Transcription Factor ELK1 (ELK1) and RSK1p90 have been recognized to be related to cell growth and differentiation related to hematopoietic function[28]. After 24-h incubation, the enhanced levels of c-Myc, ELK1 and P-RSK1p90 were noted in K562 (*p* < 0.01) (Fig. 1e) and CHRF cells (*p* < 0.001) (Fig. 1f) treated with ZQFZ at 100 µg/mL.

3.4 Protection of ZQFZ on hematopoiesis in mice with hematopoietic dysfunction

Four-week treatment with ZQFZ and rhG-CSF markedly increased the body weights of CTX-injected hematopoiesis damaged mice ($p < 0.05$) and reversed the pathological changes of organ indexes, including those of thymus ($p < 0.05$), spleen ($p < 0.01$), and liver ($p < 0.05$) (Table S2). The function of bone marrow can be reflected by the concentration of blood cells in the peripheral blood[29]. In mice with hematopoietic dysfunction, ZQFZ led to a remarkable enhancement in the concentrations of mean corpuscular HGB ($p < 0.05$), lymphocytes ($p < 0.05$), HGB ($p < 0.01$) and neutrophils ($p < 0.001$), and to a reduction in the concentrations of monocytes (MO) ($p < 0.001$) in the peripheral blood of hematopoiesis damaged mice. ZQFZ alone failed to influence body weights, organ indexes, and the cell concentrations numbers of in peripheral blood in healthy mice (Table S2 and Table 2).

	CTRL	CTX 100mg/kg					
		-	rhG-CSF(μ g/kg)		ZQFZ (g/kg)		
			22.5	0.1	0.3	0.9	0.3
NE (%)	16.5 \pm 0.7	12.8 \pm 1.2 [#]	25 \pm 9.0	40.7 \pm 2.6 ^{***}	44.8 \pm 4.4 ^{***}	37.1 \pm 2.0 ^{***}	18.2 \pm 5.4
LY (%)	54.6 \pm 8.2	17.8 \pm 4.0 ^{###}	16.5 \pm 4.0	19.0 \pm 7.6	29.8 \pm 1.5 [*]	32.2 \pm 6.0 [*]	51 \pm 6.7
MO (%)	26.9 \pm 4.0	65.7 \pm 4.8 ^{###}	61.1 \pm 6.1	38.8 \pm 8.3 ^{**}	34.1 \pm 5.2 ^{***}	30.2 \pm 5.7 ^{***}	23.7 \pm 5.3
HGB (g/L)	148.3 \pm 6.8	127 \pm 3.6 ^{##}	125 \pm 9.0	114.3 \pm 12.1	128 \pm 7.5	141.7 \pm 2.5 ^{**}	149 \pm 4.6
MCV (fL)	44.3 \pm 1.7	42.6 \pm 1.6	41.1 \pm 1.8	41.8 \pm 0.8	42.5 \pm 0.5	43.9 \pm 1.6	43 \pm 0.7
MCH (pg)	15.1 \pm 0.2	13.5 \pm 0.3 ^{###}	13.2 \pm 0.7	13.7 \pm 0.3	14.4 \pm 0.3 [*]	15.6 \pm 0.4 ^{***}	14.7 \pm 0.4
MCHC (g/L)	336.8 \pm 7.9	317 \pm 20.1	321.5 \pm 5.1	327.7 \pm 3.5	338.3 \pm 4	356 \pm 3.6 [*]	341.7 \pm 12.3

Data are showed as the means \pm S.D. (n = 10/group) and analyzed using a one-way analysis of variance followed by Tukey's test. [#] $P < 0.05$, ^{##} $P < 0.01$ and ^{###} $P < 0.001$ vs. control group, ^{*} $P < 0.05$, ^{**} $P < 0.01$ and ^{***} $P < 0.001$ vs. model group. ZQFZ, ZhenQi FuZheng fomula, CTX, cyclophosphamide, rhG-CSF, recombinant human granulocyte colony-stimulating factor. NE, Norepinephrine, LY, lymphocyte, MO, monocytes, HGB, hemoglobin, MCV, erythrocyte mean corpuscular volume, MCH, mean corpuscular hemoglobin, MCHC, mean corpuscular hemoglobin concentration.

Table 2

The effects of ZQFZ on peripheral blood cells of CTX-injected mice with hematopoietic dysfunction

CD45⁺CD19⁺ is used to characterize the B lymphocyte phenotype of BMMNCs[20]. 20.42% \pm 1.22% of CD45⁺CD19⁺ positive cells in the BMMNCs of control mice and 0.5% \pm 0.11% of CD45⁺CD19⁺ positive cells in the BMMNCs of mice were noted, respectively ($p < 0.001$) (Fig. 2a and Fig.S3a). ZQFZ and rhG-CSF significantly increased the proportion of CD45⁺CD19⁺ positive cells to 17.92% \pm 1.85% ($p < 0.001$) and 11.36% \pm 1.88% ($p < 0.001$), respectively (Fig. 2a and Fig.S3a). Another biomarker, Lin⁻, is applied to screen juvenile cells to characterize

the differentiation potential of ZQFZ on bone marrow cells[30, 31]. Extremely low percentages of juvenile cells, in the total BMMNCs were noted in hematopoiesis damaged mice ($14.87\% \pm 4.30\%$) compared with those of control mice ($52.48\% \pm 5.22\%$) ($p < 0.001$) (Fig. 3b and Fig.S3b), while ZQFZ and rhG-CSF strongly reversed this damage on cell differentiation in hematopoiesis damaged mice ($p < 0.05$, Fig. 3b and Fig.S3b).

The results of H&E staining showed that there were reductions in the numbers of cells and the proportion of vacuoles in the bone marrow cavity (Fig. 2c), multinucleated giant cells in the spleen (arrow in Fig. 2d), and the inflammatory infiltration phenomenon in liver (arrow in Fig. 2f) were noted, which were all strongly reversed by ZQFZ. No significant changes in the kidney were seen in any experimental group (Fig. 2e).

ZQFZ alone failed to influence the production of BMMNCs (Fig. 2a and b), the bone marrow cavity (Fig. 2c), and the other organ structures (Fig. 2d, e and f) in healthy mice.

3.5 ZQFZ regulates hematopoietic cytokines, especially M-CSF, related to Nrf2 and NF- κ B signaling

Forty cytokines related to the hematopoiesis were detected in the spleen tissues of mice with hematopoietic dysfunction. Compared with vehicle-treated model mice, ZQFZ affected concentrations of 19 cytokines in the spleen by $> 20\%$, 17 of which showed reduced concentrations, and the concentrations of two cytokines were increased (Fig. 3a and b; Table S3). According to a high-throughput splenic antibody chip analysis, ELISA was further applied to confirm the changes in cytokines. Compared with vehicle-treated model mice, ZQFZ enhanced the concentrations of M-CSF ($p < 0.05$), IL-2 ($p < 0.01$), and IL-5 ($p < 0.01$) and reduced the concentrations of TNF- α ($p < 0.05$), MCP-1 ($p < 0.05$), MIP-1 α ($p < 0.05$), and MCP-5 ($p < 0.01$) in the serum and the spleen (Table 3); meanwhile, ZQFZ reduced the concentrations of ROS ($p < 0.001$) in the spleen (Fig.S3c). However, rhG-CSF failed to affect the concentrations of MIP-1 α and MCP-1 in the serum and in the spleen (Table 3). Compared with healthy mice, ZQFZ failed to influence the splenic concentrations of the detected cytokines except for IL-2 ($p < 0.01$), and IL-5 ($p < 0.001$), and the serum concentrations of the detected cytokines, except for those of IL-5 ($p < 0.01$) (Table 3).

Table 3
The effects of ZQFZ on cytokines of CTX-injected mice with hematopoietic dysfunction

		CTRL	CTX 100 mg/kg					
			-	rhG-CSF	ZQFZ (g/kg)			
					22.5 (μ g/kg)	0.1	0.3	0.9
spleen	IL-2 (pg/mg)	218.9 \pm 18.0	190.3 \pm 38.6 [#]	228.8 \pm 35.0 [*]	287.2 \pm 47.8 ^{***}	286.5 \pm 23.7 ^{***}	257.6 \pm 42.3 ^{**}	258.7 \pm 35.0 ^{##}
	IL-5 (pg/mg)	4.9 \pm 0.4	3.4 \pm 1.3 ^{##}	5.6 \pm 0.8 ^{***}	6.2 \pm 0.4 ^{***}	5.7 \pm 0.7 ^{**}	5.6 \pm 0.6 ^{**}	7.0 \pm 0.8 ^{###}
	TNF- α (pg/mg)	127.3 \pm 12.2	146.0 \pm 15.3 ^{##}	132.4 \pm 15.3 [*]	137.9 \pm 14.9	134.7 \pm 9.6	126.4 \pm 9.1 ^{**}	138.8 \pm 15.2
	M-CSF (pg/mg)	38.6 \pm 6.6	37.2 \pm 4.0	48.1 \pm 5.3 ^{***}	55.7 \pm 6.7 ^{***}	44.1 \pm 4.4 ^{**}	51.0 \pm 4.6 ^{***}	45.5 \pm 5.5
	MCP-1 (pg/mg)	35.5 \pm 5.6	50.5 \pm 4.6 ^{###}	47.8 \pm 4.0	38.2 \pm 6.3 ^{**}	42.9 \pm 3.9 ^{**}	40.0 \pm 4.1 ^{***}	39.4 \pm 5.8
	MCP-5 (pg/mg)	5.5 \pm 0.5	6.9 \pm 0.5 ^{###}	6.3 \pm 0.6 [*]	5.7 \pm 0.7 ^{**}	5.1 \pm 0.4 ^{***}	5.6 \pm 0.5 ^{***}	6.0 \pm 0.5
	MIP-1 α (pg/mg)	11.0 \pm 0.6	13.6 \pm 1.2 ^{##}	13.6 \pm 1.0	12.3 \pm 0.9	11.5 \pm 0.6 ^{**}	11.8 \pm 1.3 [*]	11.7 \pm 0.7
serum	IL-2 (pg/ml)	439.4 \pm 46.4	279.2 \pm 113.0 ^{##}	293.5 \pm 45.8	446.3 \pm 77.6 ^{**}	459.2 \pm 60.8 ^{**}	479.7 \pm 112.3 ^{**}	403.7 \pm 71.0
	IL-5 (ng/l)	13.6 \pm 1.4	10.0 \pm 2.7 ^{##}	15.4 \pm 2.5 ^{**}	10.7 \pm 1.1	15.7 \pm 1.1 ^{**}	14.5 \pm 1.9 ^{**}	16.6 \pm 1.9 ^{##}
	TNF- α (ng/l)	609.9 \pm 65.2	677.6 \pm 46.2 ^{##}	654.4 \pm 36.4	625.8 \pm 44.9 [*]	627.6 \pm 44.6 [*]	583.1 \pm 73.9 ^{**}	575.2 \pm 57.2
	M-CSF (ng/l)	49.5 \pm 7.6	44.3 \pm 5.2	60.1 \pm 14.1 ^{**}	46.3 \pm 7.8	52.2 \pm 6.9 [*]	51.4 \pm 4.3 ^{**}	48.1 \pm 7.6
	MCP-1 (ng/l)	37.2 \pm 3.9	43.2 \pm 6.2 ^{##}	37.3 \pm 6.6	36.7 \pm 5.1 [*]	40.2 \pm 3.1	37.5 \pm 3.9 [*]	40.6 \pm 4.1
	MCP-5 (ng/l)	22.3 \pm 1.1	21.9 \pm 1.3	24.6 \pm 2.1 [*]	23.3 \pm 3.8	23.7 \pm 2.3	18.1 \pm 2.1 ^{**}	24.6 \pm 2.6
	MIP-1 α (ng/l)	75.1 \pm 6.9	86.3 \pm 4.0 ^{##}	81.9 \pm 6.2	73.9 \pm 6.3 ^{**}	59.8 \pm 6.7 ^{***}	76.0 \pm 5.6 ^{**}	71.4 \pm 5.0

Data are expressed as mean \pm S.D. (n = 10/group) and analyzed using a one-way analysis of variance followed by Tukey's test. [#]*P* < 0.05, ^{##}*P* < 0.01 and ^{###}*P* < 0.001 vs. control group, ^{*}*P* < 0.05, ^{**}*P* < 0.01 and ^{***}*P* < 0.001 vs. model group. ZQFZ, ZhenQi FuZheng formula, CTX, cyclophosphamide, rhG-CSF, recombinant human granulocyte colony-stimulating factor.

ZQFZ dosed at 0.9 g/kg strongly up-regulated the levels of Nrf2 ($p < 0.01$), Heme Oxygenase 1 (HO-1) ($p < 0.01$), Superoxide Dismutase-2 (SOD-2) ($p < 0.01$), SOD-1 ($p < 0.001$), P-NF- κ B ($p < 0.001$), and M-CSF ($p < 0.001$) (Fig. 3c) in the spleens of hematopoiesis damaged mice.

In primary cultured bone marrow cells, ZQFZ strongly enhanced the expressions of Nrf2 ($p < 0.001$), SOD-1 ($p < 0.001$), SOD-2 ($p < 0.001$), HO-1 ($p < 0.01$), P-extracellular regulated protein kinases (ERK)1/2 ($p < 0.001$), P-Mammalian Target of Rapamycin (mTOR) ($p < 0.001$), P-IKK $\alpha + \beta$ ($p < 0.001$) and P-NF- κ B ($p < 0.001$), and reduced the expression level of P-p38 ($p < 0.05$) (Fig. 4a).

In Nrf2-siRNA transfected K562 cells, the enhancing effects of ZQFZ on the expression levels of Nrf2 ($p < 0.01$), P-NF- κ B ($p < 0.001$), M-CSF ($p < 0.001$), P-RSK1p90 ($p < 0.05$), ELK1 ($p < 0.01$) and c-Myc ($p < 0.001$) were all strongly suppressed relative to the ZQFZ-treated normal K562 cells (Fig. 4b).

4. Discussion

To the knowledge, we first report on the effects of ZQFZ on immune and hematopoietic functions in mice. In mice with CTX-induced immune suppression, ZQFZ enhanced the activities of NK cells, and regulated immune-related factors such as ILs and chemotactic factors. Immunoglobulins protect the body from infections and can enhance humoral immunity and the body's hematopoietic function[32, 33]. Excessive production of ROS can affect the activity of inflammation-related proteins and damage the immune system[34]. Among all detected ILs, IL-2, the most comprehensively studied IL, can stimulate the growth of T and B cells, enhance the cytotoxic activities of NK cells, regulate the differentiation of Th1 cells, and further promote the release of IL-6, -10 and -12[35, 36].

A dynamic balance among the differentiation of red blood cells, the proliferation of HSCs, and the formation of blood cells, is required for regular hematopoiesis[37, 38]. The reduced self-renewal on HSCs is responsible for long-term bone marrow damage[39]. As reported, the normal function of the hematopoietic system is partially maintained by HSCs; thus, the protection and improvement of the self-renewal of HSCs may be key to develop new agents for anti-myelosuppression[39]. It was thus notable that ZQFZ enhanced the proliferation on both of K562 and CHRF cells, promoted the differentiation of K562 cells into erythrocyte lineage cells, and enhanced the levels of c-Myc, ELK1, and P-RSK1-p90 in both K562 and CHRF cells. In hematopoiesis damaged mice, ZQFZ enhanced the percentage of CD19⁺ CD45⁺ BMMNCs and the proportion of Lin⁻ in BMMNCs. The synergistic effect of CD45 and B cell receptors are involved in the activation of B lymphocytes, which are derived from mature cells in HSCs and bone marrow[40]. Meanwhile, CD19 controls the differentiation and maturation of B lymphocytes helping to maintain the humoral immunity of the human body[41, 42]. Severe autoimmune disease, such as hematopoietic dysfunction can be caused by abnormalities in the immune system[43]. Lin⁻, a specific marker for juvenile cells, contributes to cell proliferation and differentiation[31, 44]. ZQFZ enhanced the number proportion of bone marrow cells in CTX-damaged bone marrow in mice. The concentration of blood cells in peripheral blood reflects the hematopoietic function of bone marrow[29], and the pathological alterations to peripheral blood caused by CTX were all strongly restored by ZQFZ administration. All of the data confirm the role of ZQFZ in improving hematopoietic function in mice.

Cytokines, such as ILs and IFNs, regulate the proliferation and differentiation of hematopoietic cells[45, 46]. IL-2 helps to promote the expression of multiple antigens and antibodies, including IL-5, TNF- β , and CSF[36]. IL-5 regulates the activation of hematopoietic cells[47]. The host cells of a hematopoietic system damaged by long-term chemotherapy/radiotherapy releases proinflammatory cytokines, including TNF- α , that directly inhibit

hematopoietic function by suppressing the number of bone marrow precursors and inhibiting the activity of HSCs[48]. It is encouraging to note that ZQFZ enhanced the concentrations of M-CSF and ILs in the sera of hematopoiesis damaged mice. Meanwhile, ZQFZ reduced the concentrations of TNF- α , MCP-1, MCP-5 and MIP-1 α , which have been considered to suppressed the proliferation of myeloid progenitor cells. As a hematopoietic growth factor, derived from fibroblasts, M-CSF shows an important role in stimulating the differentiation and proliferation of neutrophilic precursor cells and/or bone marrow progenitor cell, and in increasing the myeloid commitment at the HSC level[49]. M-CSF can reduce the concentration of TNF- α and enhance the concentrations of IL-1, -6, and -10[50, 51]. MCP-1, MCP-5, and MIP-1 α inhibit the proliferation and maturation of HSC by limiting the expression of M-CSF under the combined action of various growth factors[52, 53]. M-CSF can promote the expression of c-Myc[54], RSK, and ETS by indirectly influencing the activation of ERKs[55, 56]. The current data suggest that enhancement of M-CSF is involved in ZQFZ-mediated enhancement on hematopoietic function.

NF- κ B signaling acts as the major modulator in inflammatory responses and hematopoiesis, such as the survival of HSCs and HSPCs and the differentiation of hematopoietic precursors[57]. In a classical signaling pathway, phosphor-mTOR promotes the phosphorylation of IKK α + β , leading to the activation of NF- κ B[58], which is translocated into the nucleus, where it binds to the enhancer region of M-CSF[59, 60]. The reduction of ROS synergistically promotes phosphor-mTOR to further regulate the activation of NF- κ B[61]. In contrast, ROS promote the expression of Nrf2 with phosphor-ERK[62]. As a feedback, the activated Nrf2 signaling enhances the expression of SOD1, SOD2, and HO-1, which help to inhibit the over-production of ROS[63]. Consequently, the activated Nrf2 signaling promotes the levels of M-CSF by up-regulating the expression of SOD1 and HO-1[64, 65]. According to a previous study, in K562 cells, the anti-inflammatory flavone wogonin inhibited Nrf2 signaling via NF- κ B inactivation[66]. We were interested to note that the regulatory effects of ZQFZ on the phosphorylation of NF- κ B, the expression of M-CSF and erythroid differentiation related proteins were significantly reversed in Nrf2-siRNA-transfected K562 cells. Based on our data, both Nrf2 and NF- κ B appear to be involved in regulating the beneficial effect of ZQFZ on hematopoietic function in mice.

There are limitations to this study. A non-dose-dependent effect of ZQFZ, which contains multiple effective components, was noted in some of our experiments, which is common for herbal medicines. More experiments will be performed to reveal the effective constituents in ZQFZ. In this present study, we reported the improvement induced by ZQFZ on immune and hematopoietic functions; however, the pharmacological mechanisms, especially the link between the two functions, still require deep examination. Furthermore, the finding of several studies suggest that an enhanced level of Nrf2 expression helps to reduce the phosphorylated activation of NF- κ B[67], and it was found in another study that the activation of NF- κ B was unaffected by Nrf2[68]. In our study, although we found that low expression of Nrf2 helped to suppress the phosphorylation of NF- κ B in Nrf2-siRNA transfected K562 cells, the relationship between NF- κ B signaling and Nrf2 signaling still requires further investigation. Due to its complex composition, ZQFZ may not directly influence the expression and/or activation between Nrf2 and NF- κ B during its enhancement of hematopoietic function.

5. Conclusion

ZQFZ prevents CTX-induced damage to immune and hematopoietic function via increasing the serum concentration of M-CSF, at least partially via altering Nrf2 and NF- κ B signaling, which supports the use of ZQFZ as an anti-myelosuppressive agent.

Abbreviations

ATCC, American Type Culture Collection; **BMMNCs**, bone marrow mononuclear cells; **BMNCs**, bone marrow nucleated cells; **BSA**, bovine serum albumin; **CTX**, cyclophosphamide; **D.D.**, double distilled; **DMEM**, Dulbecco's Modified Eagle Medium; **ELK1**, ETS Transcription Factor ELK1; **ERK**, extracellular regulated protein kinases; **FBS**, fetal bovine serum; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **HSCs**, hematopoietic stem cells; **HGB**, hemoglobin; **HO-1**, Heme Oxygenase 1; **HPCs**, hematopoietic progenitor cells; **HRP**, horseradish peroxidase; **H&E**, hematoxylin-eosin; **IFN**, Interferon; **Ig**, immunoglobulin; **IKK $\alpha+\beta$** , inhibitor of nuclear factor kappa-B kinase alpha + beta; **IL**, Interleukin; **Keap1**, Kelch-like ECH-associated protein 1; **LDH**, lactate dehydrogenase; **LY**, lymphocyte; **MCH**, mean corpuscular hemoglobin; **MCHC**, mean corpuscular hemoglobin concentration; **MCP**, monocyte chemoattractant protein; **MCV**, erythrocyte mean corpuscular volume; **M-CSF**, macrophage colony-stimulating factor; **MIP-1 α** , macrophage inflammatory protein 1 α ; **MO**, monocytes; **mTOR**, Mammalian Target of Rapamycin; **NF- κ B**, nuclear factor-kappa B; **NK**, natural killer; **Nrf2**, nuclear factor-E2-related factor 2; **PBS**, phosphate-buffered saline; **PMS**, phenazine methosulfate; **rhG-CSF**, recombinant human granulocyte colony-stimulating factor; **ROS**, reactive oxygen species; **RPMI**, Roswell Park Memorial Institute; **RSK1p90**, p90 ribosomal S6 kinases 1; **SOD**, Superoxide Dismutase; **SPF**, specific pathogen-free; **TFO**, transfer factor oral liquid; **TNF**, tumor necrosis factor; **XTT**, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; **ZQFZ**, ZhenQi FuZheng granules;

Declarations

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Authors' contributions

DW, DL and RJL conceived and performed the experiments, designed and revised the manuscript. QC, BD, SW, LL, JH and XW performed the experiments, contributed to the analysis of data and writing of the manuscript.

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Availability of data and materials

All data generated and analyzed during the present study are included in this published article. The datasets used in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animals work was strictly in accordance with the “Principle of Care and Use of Experimental Animals” of the 1st Hospital Affiliated to the Third Military Medical University (NIH publication number 8023, revised 1978).

Consent for publication

Not applicable.

Competing interests

The authors have declared that there is no conflict of interest.

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Figures

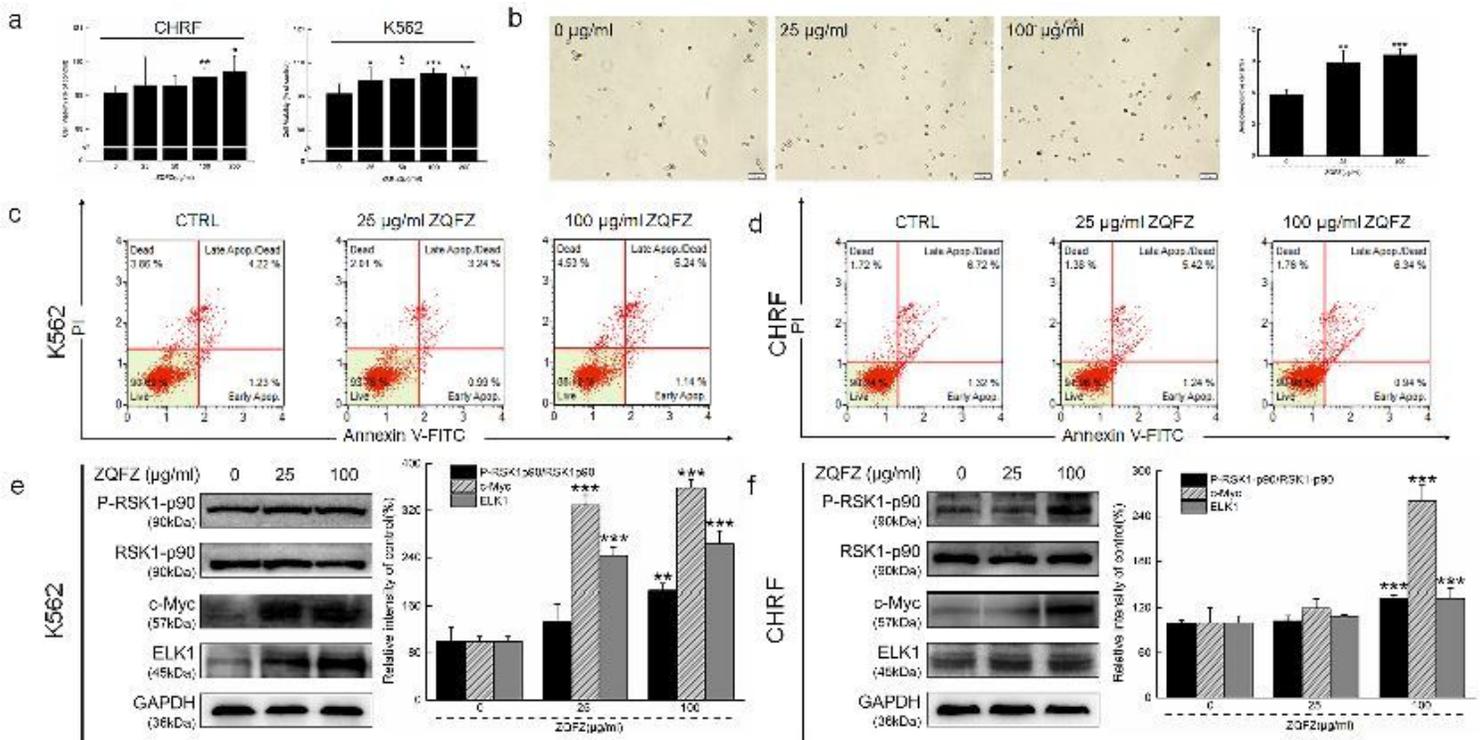


Figure 1

Potential properties of ZQFZ on the proliferation and differentiation of CHRF and/or K562 cells. Cells underwent a 24h incubation with ZQFZ at doses of 0 to 200 µg/ml. (a) ZQFZ enhanced cell proliferation of CHRF and K562 cells (n = 6). (b) ZQFZ promoted erythroid differentiation of K562 cells analyzed by benzidine staining (10×; scale bar, 100 µm; n = 6). (c) ZQFZ showed no effects on the cell apoptotic rate of K562 and (d) CHRF cells detected by Annexin V/PI staining (n = 6). ZQFZ enhanced the expression levels of P-RSK1p90, c-Myc and ELK1 in (e) K562 cells and (f) CHRF cells detected by Western blotting (n = 6). Quantitative data of protein expression levels were normalized by their GAPDH expressions and are shown as percentage of corresponding relative intensity of the control cells. Data are shown as the mean ± S. D. (n = 6). *p < 0.05, ** p < 0.01 and *** p < 0.001 vs. 0 µg/ml ZQFZ-treated cells. ZQFZ, ZhenQi FuZheng formula.

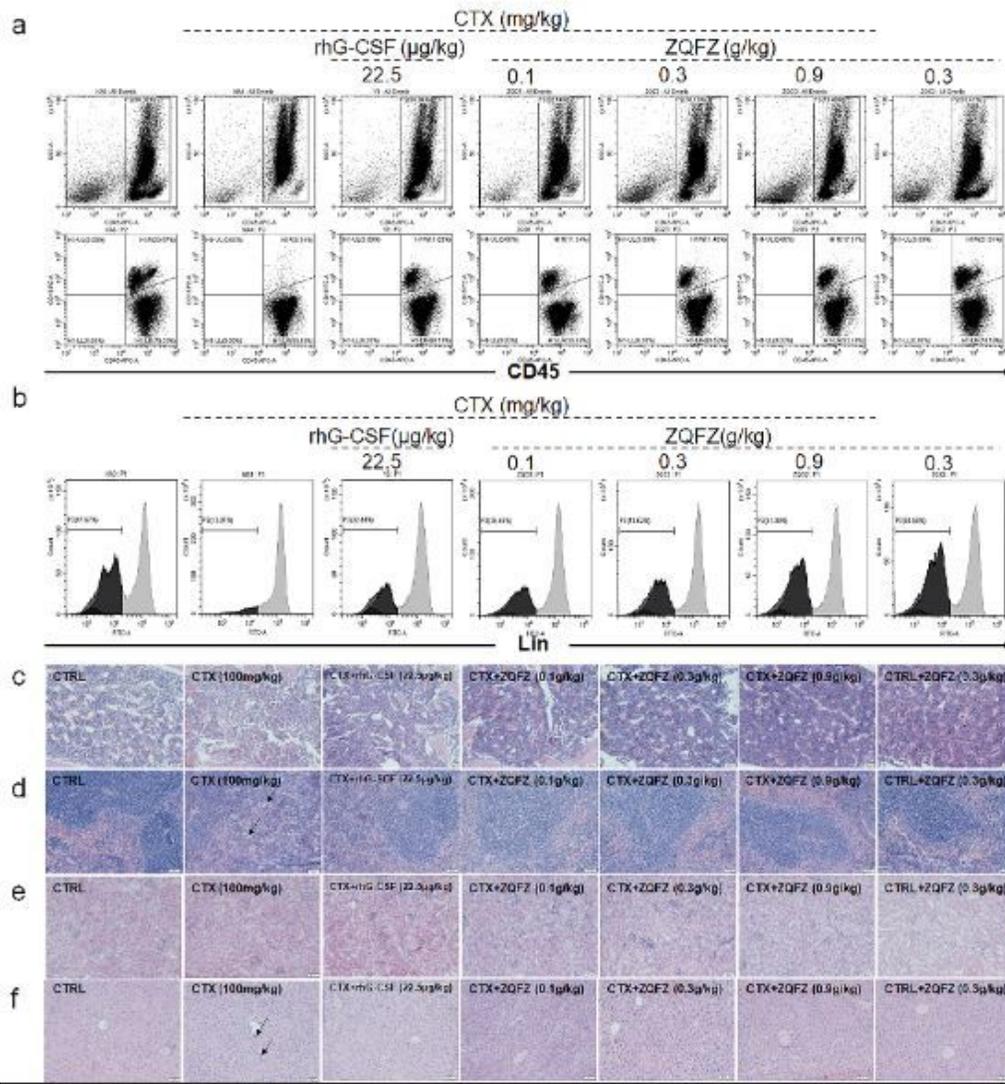


Figure 2

ZQFZ and rhG-CSF improved hematopoietic function in mice after CTX-induced damage. (a) Flow cytometry was used to analyze the proportion of leukocytes in the murine bone marrow. CD45 was used to sort leukocytes. CD45+CD19+ represents the B lymphocytes. (b) Percentage of blasts (Lin-) in the murine bone marrow of the CTX-injected mice were analyzed using a flow cytometry assay. H&E staining procedure was used to evaluate pathological alterations. (c) Cellularity of bone marrow; (d) spleen (arrows show the multinucleated giant cells); (e) kidney; and (f) liver (arrows show inflammatory infiltration phenomenon) under a light-microscope digital camera (20 \times ; scale bar, 50 μm). CTX, cyclophosphamide, rhG-CSF, recombinant human granulocyte colony-stimulating factor, ZQFZ, ZhenQi FuZheng formula.

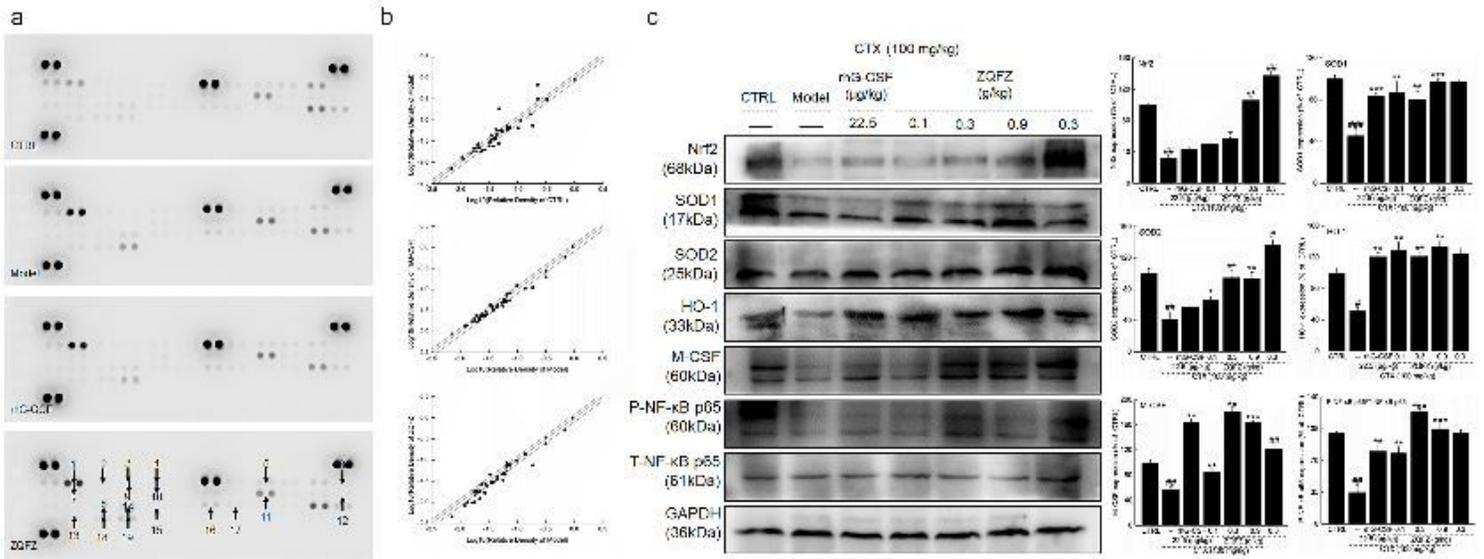


Figure 3

Effects of ZQFZ and rhG-CSF on the 40 cytokines in mice spleens were detected by use of a mouse cytokine and some of the protein involved in the protective effects of ZQFZ on mice with CTX induced hematopoietic dysfunction. Array Panel A Kit. (a) Graphical representation of cytokine expressions. Arrows indicate factors with a change of more than 20% (ZQFZ group vs. model group). 1. C5/C5a; 2. G-CSF; 3. GM-CSF; 4. I-309; 5. IL-1 α ; 6. IL-2; 7.IL-4; 8. IL-5; 9. IL-6; 10. IL-7; 11. IL-16; 12. IL-27; 13. I-TAC; 14. M-CSF; 15. JE; 16. MIG; 17. MIP-1 α ; 18. TNF- α ; 19. TIMP-1, (b) Scatter diagram of the 40 cytokines. The relative density is the ratio of the absolute value and the reference spot value; (c) ZQFZ regulated the expression levels of Nrf2, SOD1, SOD2, HO-1, P-NF- κ B p65 and M-CSF in the spleen of CTX-injected mice. The quantitative data of the expression levels of target proteins were normalized by the corresponding GAPDH and the related total protein expressions. Data are shown as mean \pm S. D. (n = 6) and analyzed using a one-way ANOVA. # p<0.05, ## p <0.01 and ### p <0.001 vs. control group, * p < 0.05, ** p < 0.01 and *** p < 0.001 vs. model group. CTX, cyclophosphamide, rhG-CSF, recombinant human granulocyte colony-stimulating factor, ZQFZ, ZhenQi FuZheng formula.

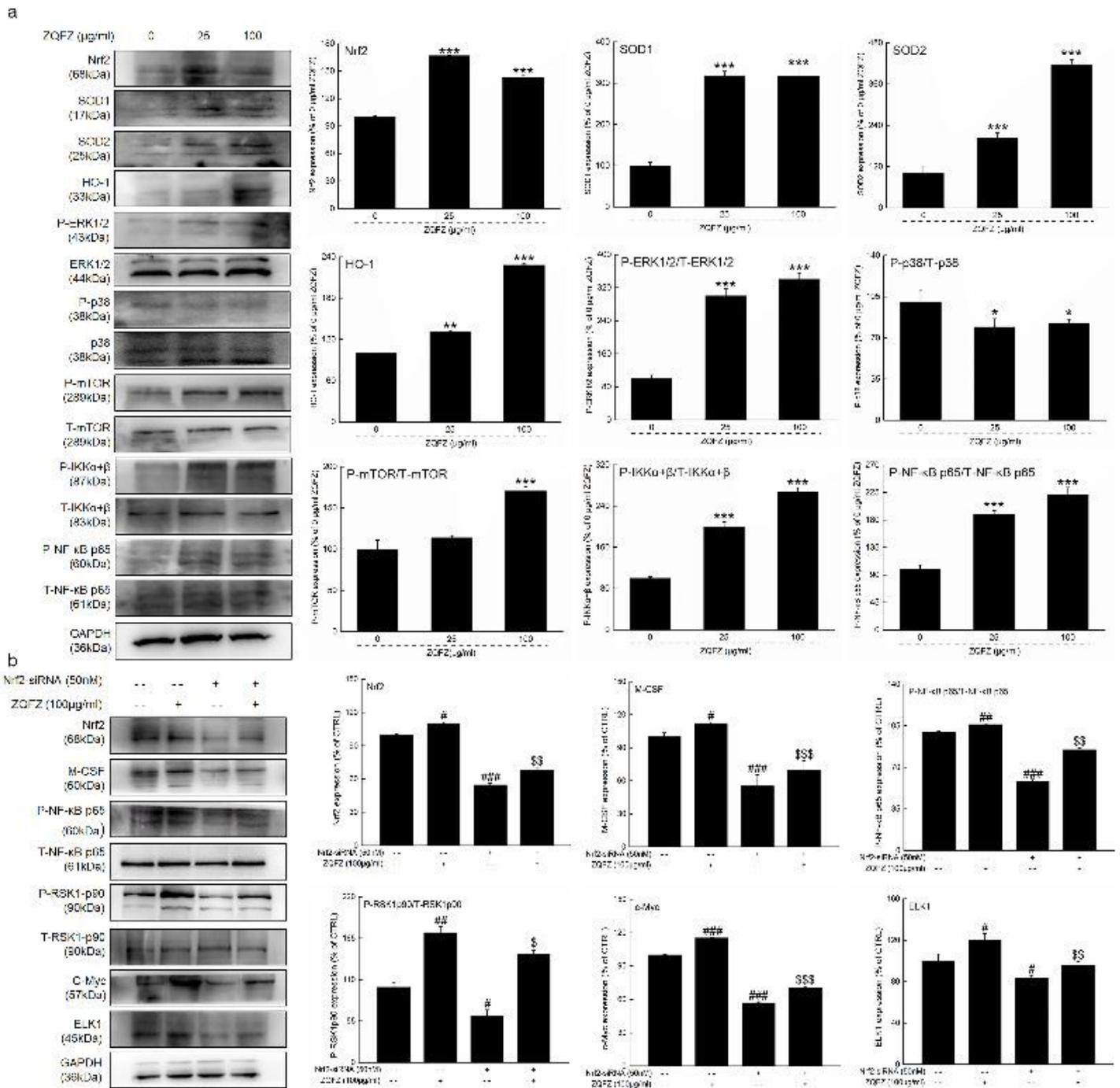


Figure 4

Effects of ZQFZ on protein expression in bone marrow cells and Nrf2-siRNA transfected K562 cells. (a) ZQFZ changed the expression levels of target proteins in primary cultured bone marrow cells, and their quantitative data for P-ERK1/2, P-p38, Nrf2, SOD1, SOD2, HO-1, P-mTOR, P-IKKα+β and P-NF-κB p65 levels were normalized by the corresponding GAPDH and related total protein expressions. (b) Nrf2-siRNA transfection strongly abolished the effects of ZQFZ on the protein expressions in K562 cells. Up-regulation of ZQFZ on the expressions of Nrf2, M-CSF, P-NF-κB p65, P-RSK1p90, c-Myc and ELK1 were strongly abolished in Nrf2-siRNA transfected K562 cells. Quantification data of proteins were normalized by corresponding GAPDH. Data are shown as mean ± S. D. (n = 6) and analyzed by one-way ANOVA. # p < 0.05, ## p < 0.01 and ### p < 0.001 vs. control cells, \$ p < 0.05,

$p < 0.01$ and

\$ p < 0.001 vs. ZQFZ-exposed K562 cells. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs. 0 µg/ml ZQFZ-treated cells. ZQFZ, ZhenQi FuZheng formula.

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