J Ginseng Res 44 (2020) 291–299

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research Article

Compatibility effects of ginseng and *Ligustrum lucidum* Ait herb pair on hematopoietic recovery in mice with cyclophosphamide-induced myelosuppression and its material basis

Jiahong Han ☆, Min Dai ☆, Yan Zhao, Enbo Cai*, Lianxue Zhang**, Xiaohuan Jia, Nian Sun, Xuan Fei, Hui Shu

College of Chinese Medicinal Materials, Jilin Agricultural University, Changchun, China

ARTICLE INFO

Article history:
Received 4 November 2018
Accepted 7 January 2019
Available online 11 January 2019

Keywords:
Chemotherapy
Ginseng
Ginseng–*Ligustrum lucidum* Ait herb pair
Material basis
Myelosuppression

ABSTRACT

Background: Ginseng (G) and *Ligustrum lucidum* Ait (LLA) are core traditional Chinese medicines in treating myelosuppression formula. The present study was designed to profile effect of G and LLA herb pair (G-LLA) on myelosuppressed mice.

Methods: The mice myelosuppression model was established by intraperitoneal (i.p.) injection of cyclophosphamide (Cy). Hematopoietic function of bone marrow was measured by hemopoietic progenitor cell culture and peripheral blood count, and serum hemopoietic factors were tested by enzyme-linked immunosorbent assay. Bone marrow cell cycle was performed by flow cytometry. HPLC was used to measure 20 potential chemical components related to myelosuppression, including ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2, Rb_3, Rd, Rk_3, Rh_4, 20 (S)-Rg_3, 20 (R)-Rg_3, Rk_1, Rg_5, salidroside, and so on.

Results: G, LLA, and G-LLA improved the amount of peripheral blood cells and bone marrow cells of myelosuppressed mice ($P < 0.01$). They significantly increased the colony quantity of colony-forming unit–granulocyte macrophage, burst-forming unit–erythroid, colony-forming unit–erythroid, and colony-forming unit–megakaryocyte and amount of G_2/M and S phase cells ($P < 0.01$). They also significantly decreased the amount of hematopoiesis-related cytokines ($P < 0.01$). The content of chemical components in G-LLA changed, and the change of rare saponin was the most obvious.

Conclusion: These results show that G-LLA herb pair might produce synergistic or complementary compatibility effects on bone marrow suppression after chemotherapy. It suggests that the substance basis of G-LLA for treating bone marrow suppression may be effective chemical components.

1. Introduction

Cancer is a broad group of diseases involving unregulated cell growth. In cancers, cells divide and grow uncontrollably, forming malignant tumors, which may invade nearby parts of the body [1]. It is recognized internationally as one of the major diseases leading to human death, seriously threatening human life and social development. Chemotherapeutic drugs are essential tools in malignancy treatment [2]. Cy is one of the alkylating agents that are used most extensively [3]. However, its treatment is often accompanied by serious side effects [4]. Myelosuppression, for one, is serious in that it may cause serious morbidity and mortality problems [5], making stimulation of hematopoiesis an important issue in clinical cancer treatments. After high doses of chemotherapy, the patients almost invariably suffer severe pancytopenia. The severe neutropenia resulted acquisition of life-threatening infections. Postchemotherapy-marked thrombocytopenia may lead to serious bleeding. Significant pancytopenia may also delay onset of the next cycle of chemotherapy [6,7].

Traditional Chinese Medicines (TCMs) have been used for thousands of years in China. Their less side effect, proven efficacy, and low cost have been considered valuable as complementary or

* Corresponding author. College of Chinese Medicinal Materials, Jilin Agricultural University, 2888 Xincheng Street, Changchun, Jilin province, 130118, China.
** Corresponding author. College of Chinese Medicinal Materials, Jilin Agricultural University, Xincheng Street No. 2888, Changchun, Jilin province, 130118, China.
E-mail address: caienbo126621@126.com (E. Cai).
☆ Jiahong Han and Min Dai contributed equally to this work.

https://doi.org/10.1016/j.jgr.2019.01.001
p1226-8453 e2093-4947/$ – see front matter

alternative medicines for health care and treatment of diseases worldwide [8,9]. Chinese herbal formulas are the main form of TCM clinical application [10]. Herb pairs are the minimum units of TCM formulae, which are formed by only two herbs. It is not a random combination of two herbs, nor is the simple accumulation of efficacy, but the simple and delicate experience of ancient Chinese medicine practitioners. Essentially, herb pairs exert the role of reducing toxicity and enhancing efficacy by drug–drug interaction [11]. Owing to the functional characteristics of multicomponents and multitargets [12], studying the material basis of herb pair can help to analyze the theory of TCM compatibility and its pharmacological action mechanism [13].

Ginseng (G) has been used as a therapeutic medicine for thousands of years. Its traditional function is to replenish qi. Now, research shows that it may be useful for treating cardiovascular diseases, cancers, tumors, and chronic metabolic syndromes such as diabetes [14–16]. The most pharmaceutically active constituents found in G is ginsenoside [17]. *Ligustrum lucidum* Ait (LLA) is a Yin tonic. It has immunomodulatory and antitumor effects [18]. One of the main active ingredients is salidroside [19]. These two TCMs are widely used in prescriptions for treating myelosuppression, for example, Sijunzi decoction [20]and Buxue Shengbai decoction [21]. Antitumor Shengbai tablet is also involved in these two TCMs [22]. G and LLA herb pair can invigorate qi and supplement blood and nourishing yin, both qi and blood, Yin and Yang.

Based on the aforementioned fact, we inferred that the herb pair G and LLA (G-LLA) may result in an improved bone marrow hematopoietic function. To test our hypothesis, we studied the effects of G, LLA, and G-LLA on hematopoietic function of bone marrow, including peripheral blood and bone marrow nucleated cell (BMNC) counts, and the immune organs in Cy-induced myelosuppression mice. Meanwhile, we detected the proliferation and differentiation of hematopoietic progenitor cells, hematopoiesis-related cytokines factor content, and bone marrow cell cycle. In addition, the material basis of G-LLA was determined by HPLC.

2. Materials and mathodes

2.1. Chemicals and materials

Ginsenoside Rg_1, Re, Rf, Rb_1, Rg_2, Rc, Rb_2, Rb_3, Rd, Rk_3, F_2, Rh_4, 20 (S)-Rg_3, 20 (R)-Rg_3, Rk_1, Rg_5, Rh_2, compound K, Protopanaxdiol (PPD), Protopanaxatriol (PPT), salidroside, tyrosol, hydroxytyrosol, ligustroflavone, and specnuezhenide were obtained from Aladdin (Shanghai, China) or Yuanye (Shanghai, China).

2.2. Preparation of herb extracts

G-LLA (100 g) was soaked in 1 L of water for 60 min before boiling for 60 min. An aqueous extract was obtained by filtration. Water was added and cooked twice, and then, all filtrates were combined and concentrated. Single-herb extracts were prepared in the same way, and each concentrate was stored at 4°C until use. In this study, the leaching rates of G, LLA, and G-LLA were 52%, 20%, and 35.36% respectively.

2.3. Animals and groups

Male Balb/c mice (weighing 18–22 g) were obtained from the Laboratory Animal Quality Testing Center of Jilin Province (Certificate No. SCXK-2016-0003). All mice were adapted to the new laboratory 7 days before the experiment and maintained at controlled temperature (22 ± 2°C) and humidity (50 ± 10%) with a 12-h/12-h light/dark cycle. All efforts were made to minimize the pain of animals. The animal procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of China Academy of Chinese Medical Sciences.

2.4. Treatment and experimental design

After one-week environmental adaptation period, mice were randomly divided into six groups: control, Cy, G (Ginseng), LLA (*Ligustrum lucidum* Ait), G-LLA (Ginseng and Ligustrum lucidum Ait), and positive (Zhenqi Fuzheng granules). All the groups except the control group were injected intraperitoneally with Cy (100 $mg \cdot kg^{-1}$, 0.1 $mL \cdot 10\ g^{-1}$) once daily for 3 continuous days. Positive, G, LLA, and G-LLA groups were daily administered intragastrically with equal volume Zhenqi Fuzheng granules (4.5 $g \cdot kg^{-1}$), G (0.52 $g \cdot kg^{-1}$), LLA (0.3 $g \cdot kg^{-1}$), and G-LLA (0.884 $g \cdot kg^{-1}$), respectively, at 9:00 AM for 12 days, whereas control and Cy groups received an equivalent normal saline (0.9% NaCl aq) for 12 days.

2.5. Index detection

2.5.1. Determination of peripheral blood cells

Twenty-four hours after the last administration, blood was collected on the day of sacrifice by retroorbital bleed into EDTA-K_2 tubes using an automatic blood analyzer to detect white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), and platelet (PLT).

2.5.2. Karyocyte count

Double femurs were removed under aseptic conditions, and bone marrow cells were flushed using sterile phosphate-buffered saline (PBS) to form a single-cell suspension. The suspension was placed into a centrifuge tube and centrifuged for 10 min at 1200 $r \cdot min^{-1}$. Two milliliters of RBC lysis buffer was added, let undisturbed for 3 minutes after blending, and centrifuged for 10 min at 1200 $r \cdot min^{-1}$. The bone marrow karyocytes were removed and rinsed twice with 1 mL of sterile PBS for 10 min at 1200 $r \cdot min^{-1}$ and then resuspended in sterile PBS and counted using an inverted microscope.

2.5.3. Calculation of immune organ indices

Mice were sacrificed 24 hours after the last intragastric administration. Then, the thymus and spleen of mice of each group were removed and weighed, and the following index was calculated. The spleen and thymus indexes were calculated using the following formula:

$$\text{Spleen index}\ (\%) = (\text{spleen weight} / \text{body weight}) \times 100\%$$

$$\text{Thymus index}\ (\%) = (\text{thymus weight} / \text{body weight}) \times 100\%$$

2.5.4. Bone marrow culture of colony formation

After the BMNC count was adjusted to the desired concentration, the colony-forming unit–granulocyte macrophage (CFU-GM), the colony-forming unit–megakaryocyte (CFU-Meg), colony-forming unit–erythroid (CFU-E), and burst-forming unit–erythroid (BFU-E) were cultured in a semisolid culture system using mercapto alcohol, 3% L-glutamine, horse serum, recombinant murine granlulocyte macrophage-colony stimulating factor (rmGM-CSF) ($50^{\circ}ng^{\circ}ml^{-1}$), recombinant human erythropoietin (rhEPO) ($20^{\circ}u^{\circ}ml^{-1}$), recombinant murine Interleukin-3 (rmlL-3) ($20^{\circ}ng^{\circ}ml^{-1}$), recombinant murine Thromobopoietin (rmTPO) ($5^{\circ}ng^{\circ}ml^{-1}$), BMNCs, Iscove's modified Dulbecco's medium (IMDM), and 3% agar. There were three parallel groups in each

group cultivated at 37°C, 5% CO_2, and saturated humidity. On the 4th day of culture, CFU-E cell colonies were counted under an inverted phase contrast microscope (8–32 cells were taken as one colony), and on the 8th day of culture, BFU-E cell colonies (50 cells were taken as one colony), CFU-GM cell colonies (50 cells were taken as one colony), and CFU-Meg cell colonies (3 cells were taken as one colony) were counted under an inverted phase contrast microscope.

2.5.5. Cytokine assay

The levels of cytokine GM-CSF, erythropoietin (EPO), and thrombopoietin (TPO) were determined by colorimetry according to the manufacturer-provided procedure. The colorimetry was read using an enzymatic reader using the mouse enzyme-linked immunosorbent assay kit according to the manufacturer's instructions.

2.5.6. Cell cycle

Single bone marrow cell suspension was centrifuged (1 000 r/min, 10 min). The supernatant was removed, and precipitated cells were washed twice with PBS, and then 2 mL of 70% cold ethanol was added to fix the cells overnight at 4°C. The cells were then dyed with propidium iodide staining solution (protection from light for 30 min). Then, cell cycle was detected by flow cytometry. Data of every cell cycle phase were calculated in percentage.

2.6. HPLC analysis: determination of main chemical components in G, LLA, and G-LLA

2.6.1. Sample preparation

Samples mentioned in section 2.2 were taken in a 100-ml conical flask and extracted with 50 ml of methanol at room temperature (30°C) once for 1 h. When the extraction was completed, the extract was filtrated and transferred into a 50-ml conical flask. Twenty-five milliliters of the extract was taken in an evaporating dish, and the extract was concentrated under reduced pressure of −0.09 MPa at 80°C. The residue was transferred to a 5-mL volumetric flask, diluted to the desired volume with methanol, and then filtered for HPLC analysis.

2.5.2. Establishment of chromatographic conditions

Ginsenoside analysis was carried out on a 26-201 liquid chromatograph (Alltech, US). The analytical column was an Agilent C_{18} (State of California, USA), 5 μm, 250 mm × 4.6 mm.

The separation of ginsenosides was obtained by gradient elution [eluents were a mixture of water (A) and acetonitrile (B)]. The process was performed according to the following profiles: 0–40 min, 18–21% (B); 40–42 min, 21–26% (B); 42–46 min, 26–32% (B); 46–66 min, 32–33% (B); 66–68 min, 33–34% (B); 68–73 min, 34–38% (B); 73–80 min, 38–49% (B); 80–84 min, 49% (B); 84–85 min, 49–51% (B); 85–90 min, 51–60% (B); 90–92 min, 60–65% (B); 92–99 min, 65% (B); 99–104 min, 65–85% (B); 104–111 min, 85% (B); 111–115 min, 85–18% (B); 115–122 min, 18 (B). The flow rate was kept at 1.0 mL/min. The injection volume was 10 μL. The absorbance was measured at a wavelength of 203 nm, and the column temperature was maintained at 30°C.

The separation of salidroside, tyrosol, hydroxytyrosol, ligustroflavone, and specnuezhenide was obtained by gradient elution [eluents were a mixture of water (A) and acetonitrile (B)]. The process was performed according to the following profiles: 0–10 min, 8–15% (B); 10–30 min, 15–30% (B); 30–40 min, 30–8% (B); 40–42 min, 8% (B). The flow rate was kept at 1.0 mL/min. Detection was carried out at 220 nm. The injection volume was 10 μL.

2.7. Statistical analysis

All the statistical analyses were performed using the Statistical Package for Social Sciences (SPSS (IBM, USA)), version 17.0. The quantitative data are expressed as the means ± standard deviation. All statistical comparisons were performed using a one-way analysis of variance test. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Improves general condition of myelosuppressed mice

Cy administration for 3 days adversely affected the mice compared with the control mice. Control mice were active, sleek, and eating well, maintaining normal weight, whereas Cy mice were inactive, dull, lusterless, unresponsive to stimuli, and not eating well, with shrunken back. They also displayed weight loss and thin fur, whereas G-treated mice, LLA-treated mice, or G-LLA–treated mice displayed reduced adverse effects in their appearances, fur, and general conditions. The G-LLA group showed the most obvious performance.

3.2. Increased peripheral blood cells counts of myelosuppressed mice

These results indicated that the amount of WBC, RBC, HGB, and PLT in the Cy group was significantly less than that in the control group ($P < 0.01$ or $P < 0.05$). This confirmed the successful mice model of bone marrow suppression. G, LLA, and G-LLA therapy could significantly improve peripheral blood cell counts of myelosuppressed mice. There was no significant difference in the amounts of WBC, RBC, and HGB between the G-, LLA-, and G-LLA–treated mice. However, G-LLA significantly increased PLT ($P < 0.05$) (Fig. 1)

3.3. Increased the count of BMNCs of myelosuppressed mice

As shown in Fig. 2, the BMNCs were significantly reduced in Cy group ($P < 0.01$). After administration, G-LLA significantly increased BMNCs compared with the Cy group ($P < 0.01$), and its effect was superior to G and LLA ($P < 0.01$).

3.4. Improves thymus index and spleen index of myelosuppressed mice

As shown in Table 1, the mean thymus index in the Cy mice was significantly decreased and the spleen index in the Cy mice was significantly increased compared with that in the control group ($P < 0.01$). G, LLA, and G-LLA significantly increased the thymus index, compared with the Cy group ($P < 0.01$). G-LLA–treated group showed superior thymus index compared to G- and LLA-treated groups ($P < 0.01$) and showed better spleen index than G-treated group ($P < 0.01$).

3.5. Promotes proliferation of granulocytic, erythrocyte, and megakaryocyte progenitor cells of myelosuppressed mice

Table 2 showed that CFU-GM, BFU-E, CFU-E, and CFU-Meg colony formation decreased significantly in the Cy mice ($P < 0.01$ vs. normal mice). In response to G, LLA, and G-LLA therapy for 12 days, the CFU-GM, BFU-E, CFU-E, and CFU-Meg colony numbers significantly increased ($P < 0.01$ vs. Cy mice), and G-LLA was more significant than G and LLA ($P < 0.01$).

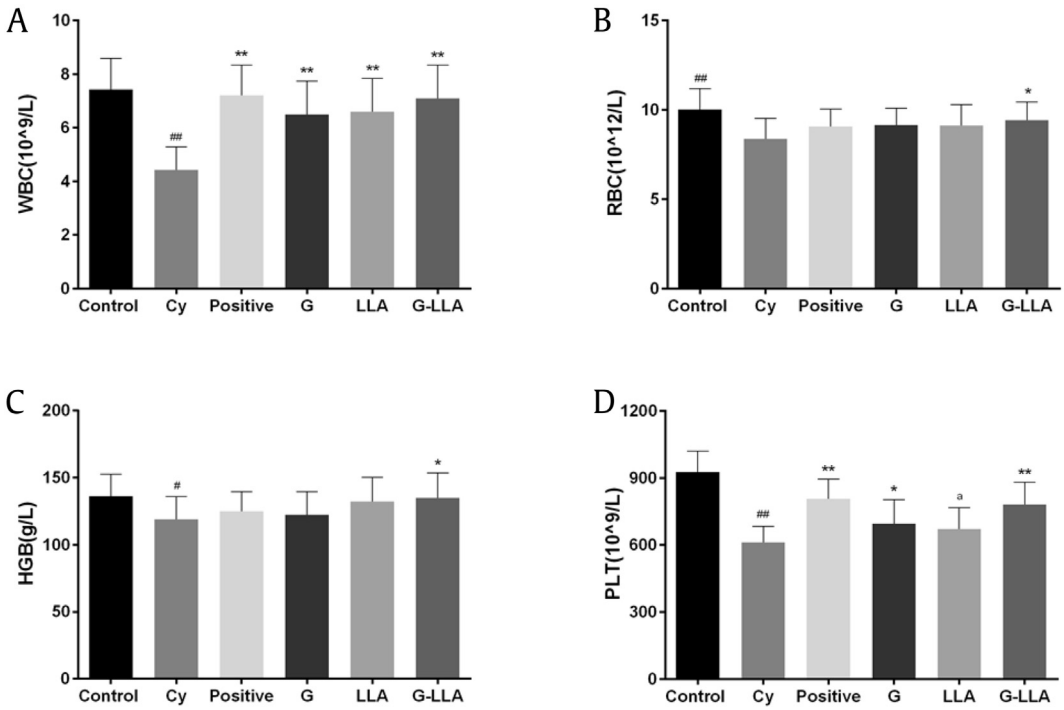

Fig. 1. Effect of G-LLA on peripheral blood cells in Cy-induced mice. (A) WBC, (B) RBC, (C) HGB, (D) PLT. Data are expressed as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs control; $^{*}P < 0.05$, $^{**}P < 0.01$, vs Cy; $^{a}P < 0.05$ vs G-LLA. Cy, cyclophosphamide; G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; HGB, hemoglobin; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

3.6. Upregulates expression of hematopoiesis-related cytokines of myelosuppressed mice

To examine Cy-induced hematopoietic cytokine production, the content of cytokines in serum was estimated by enzyme-linked immunosorbent assay (Fig. 3). Cy treatment dramatically increased GM-CSF, EPO, and TPO expression in mice. When treated with G-LLA, the release of GM-CSF and TPO decreased remarkably ($P < 0.01$), whereas there was definite improvement in the expression of EPO ($P < 0.01$).

3.7. Promotes cell proliferation of myelosuppressed mice

The ratio of G_0/G_1 phase in the Cy group increased and the ratio of S and G_2/M phase decreased after Cy administration (Fig. 4).

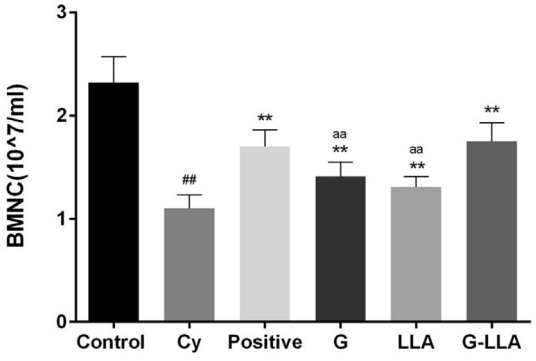

Fig. 2. Effect of G-LLA on the BMNC count in Cy-induced mice. Data are expressed as mean ± SD. $^{\#\#}P < 0.01$, vs control; $^{**}P < 0.01$, vs Cy; $^{aa}P < 0.01$ vs G-LLA. BMNC, bone marrow nucleated cell; Cy, cyclophosphamide; G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; SD, standard deviation.

Table 1
Effect of G-LLA on the thymus/spleen index in Cy-induced mice

Groups	Thymus index (mg/g)	Spleen index (mg/g)
Control	1.50 ± 0.13	4.03 ± 0.52
Cy	0.51 ± 0.08$^{\#\#}$	6.54 ± 0.89$^{\#\#}$
Positive	1.01 ± 0.12**	5.51 ± 0.72**
G	0.70 ± 0.05^{**aa}	4.94 ± 0.31^{**aa}
LLA	0.68 ± 0.08^{**aa}	6.03 ± 0.62
G-LLA	0.88 ± 0.06**	5.54 ± 0.57**

Data are expressed as mean ± SD.
Cy, cyclophosphamide; G, ginseng; LLA, *Ligustrum lucidum* Ait; SD, standard deviation.
$^{\#\#}P < 0.01$ vs control.
$^{**}P < 0.01$ vs Cy.
$^{aa}P < 0.01$ vs G-LLA.

Table 2
Effect of G-LLA on the yield of the hematopoietic stem/progenitor cells in *in vitro* colony

Groups	CFU-GM	CFU-E	BFU-E	CFU-Meg
Control	83.00 ± 9.27	106.90 ± 13.66	58.20 ± 10.25	96.90 ± 12.97
Cy	37.00 ± 6.62$^{\#\#}$	51.70 ± 8.63$^{\#\#}$	26.10 ± 4.79$^{\#\#}$	45.00 ± 7.22$^{\#\#}$
Positive	69.10 ± 6.95**	79.20 ± 8.39**	43.20 ± 6.09**	73.00 ± 6.00**
G	48.90 ± 6.05^{**aa}	71.10 ± 6.59^{**aa}	36.00 ± 4.42^{**aa}	64.10 ± 7.48^{**aa}
LLA	59.00 ± 6.38^{**aa}	71.40 ± 8.45^{**aa}	40.00 ± 5.21^{**aa}	61.10 ± 7.05^{**aa}
G-LLA	74.30 ± 8.78**	85.20 ± 7.25**	48.80 ± 6.86**	82.20 ± 7.76**

Data are expressed as mean ± SD.
BFU-E, burst-forming unit–erythroid; CFU-E, colony-forming unit–erythroid; CFU-GM, colony-forming unit–granulocyte macrophage; CFU-Meg, colony-forming unit–megakaryocyte; Cy, cyclophosphamide; G, ginseng; LLA, *Ligustrum lucidum* Ait; SD, standard deviation.
$^{\#\#}P < 0.01$ vs control.
$^{**}P < 0.01$ vs Cy.
$^{aa}P < 0.01$ vs G-LLA.

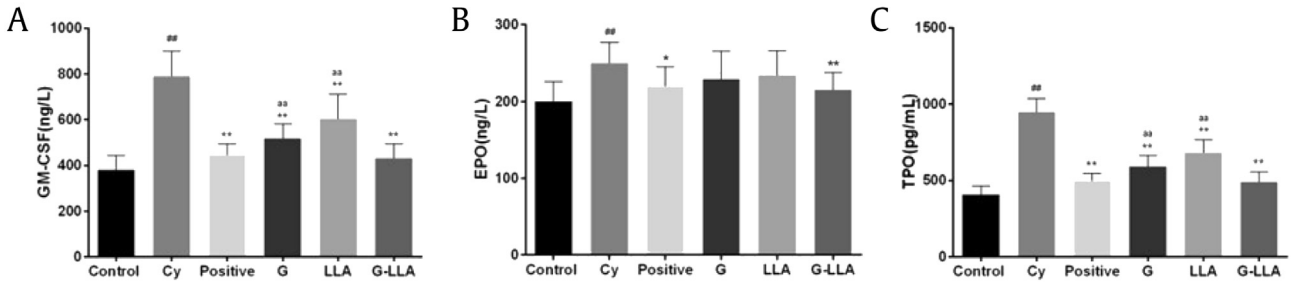

Fig. 3. Effect of G-LLA on the hematopoiesis-related cytokines. (A) GM-CSF, (B) EPO, (C) TPO. Data are expressed as mean ± SD. $^{\#\#}P < 0.01$, vs control; $^{*}P < 0.05$, $^{**}P < 0.01$, vs Cy; $^{aa}P < 0.01$ vs G-LLA. Cy, cyclophosphamide; EPO, erythropoietin; GM-CSF, granulocyte–macrophage colony-stimulating factor; G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; SD, standard deviation; TPO, thrombopoietin.

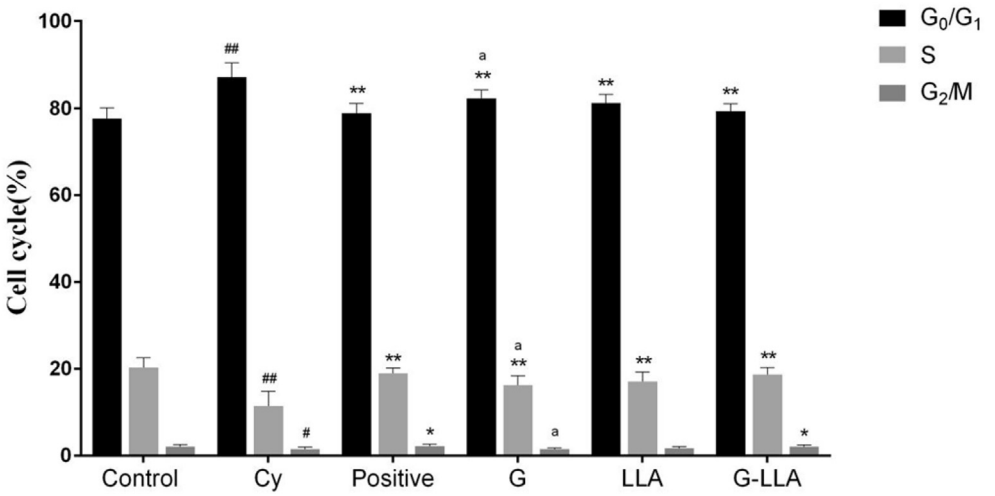

Fig. 4. Effect of G-LLA on cell cycle in Cy-induced mice. Data are expressed as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs control; $^{*}P < 0.05$, $^{**}P < 0.01$, vs Cy; $^{a}P < 0.05$ vs G-LLA. Cy, cyclophosphamide; G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; SD, standard deviation.

These changes in cell cycle phase were significant compared with the control group ($P < 0.05$ or $P < 0.01$). The percentage of G_0/G_1 phase cells in G, LLA, and G-LLA groups significantly decreased ($P < 0.01$) and S phase cells significantly increased ($P < 0.01$) compared with the Cy group. The G-LLA group showed better results than the G group ($P < 0.05$).

3.8. Changes in the content of active ingredients in G-LLA

Calibration curves were constructed from the measured peak areas (Table 3). The remarkable ginsenoside profile differences between G and G-LLA are shown in Fig. 5 and Fig. 6. As can be seen from the HPLC chromatogram, compared with G, the conventional saponins in G-LLA decreased and transformed into some rare ginsenosides. In these newly formed saponins, the content of Rg_5 is the highest, up to 1.544%, and that of 20(R)-Rg_3 increased from 0 to 0.144%.

Table 4 and Fig. 7 show the contents of the active ingredients of LLA and G-LLA. Compared with LLA, the contents of hydroxytyrosol and ligustroflavone in G-LLA were increased, whereas the contents of salidroside, tyrosol, and specnuezhenide were decreased.

4. Discussion

Modern research proves that most antitumor drugs have side effects that are diverse on the hematopoietic system. Some drugs have direct cytotoxicity to bone marrow stem cells or their

Table 3
Regression equation

Constituent	Regression equation	Linear range/(mg·mL^{-1})	R^2
Rg_1	Y = 941151X+4400.5	0.00625–0.8	0.9995
Re	Y = 1319214X+6776.2	0.00625–0.8	0.9995
Rf	Y = 2128457X+10822	0.00625–0.8	0.9997
Rg_2	Y = 2179465X+489.13	0.00625–0.8	0.9997
Rb_1	Y = 1592585X-1499.8	0.00625–0.8	0.9995
Rc	Y = 1427674X+11390	0.00625–0.8	0.9991
Rb_2	Y = 1406287X+6352.6	0.00625–0.8	0.9998
Rb_3	Y = 1047403X+4769.1	0.00625–0.8	0.9998
Rd	Y = 1489587X+7119.6	0.00625–0.8	0.9998
Rk_3	Y = 778048X+834.75	0.00625–0.8	0.9998
F_2	Y = 1844627X+9341.1	0.00625–0.8	0.9997
Rh_4	Y = 560149X+4632.2	0.00625–0.8	0.9990
20 (S)-Rg_3	Y = 2165757X-3227.3	0.00625–0.8	0.9997
20 (R)-Rg_3	Y = 2083251X+21551	0.00625–0.8	0.9991
PPT	Y = 2633345X+15096	0.00625–0.8	0.9995
Rk_1	Y = 3056413X+1370.3	0.00625–0.8	0.9998
Rg_5	Y = 475481X-2798.3	0.00625–0.8	0.9998
CK	Y = 2566303X+19093	0.00625–0.8	0.9994
Rh_2	Y = 2225003X+10720	0.00625–0.8	0.9997
PPD	Y = 3330911X+45446	0.00625–0.8	0.9996
Salidroside	Y = 7771756x+6002.1	0.01560–0.25	0.9990
Tyrosol	Y = 16039794x+24909	0.01560–0.25	0.9991
Hydroxytyrosol	Y = 11133699x-30019	0.01560–0.25	0.9990
Ligustroflavone	Y = 9472074x+1134.3	0.00195–0.03	0.9992
Specnuezhenide	Y = 5801682x+44282	0.06250–1.0	0.9991

CK, compound K.

progenitor cells, whereas others indirectly affect hematopoietic function [23]. Upon most occasions, drugs that have direct effect can lead to the reduction of circulating blood cells and in bone marrow cellularity, and when their dose is very high, it may cause irreversible reduction of the proliferative potential of hematopoietic stem cells [24]. Related experiments show that this permanent stem cell injury may cause the hematopoietic bone marrow failing to produce a sufficient number of blood cells instead which leads to myelosuppression. Therefore, myelosuppression is the most common and serious side effect of cytotoxic chemotherapy.

Myelosuppression is a decrease in the activity of blood cells in the bone marrow. Both WBC and RBC in peripheral blood are derived from stem cells in the bone marrow [25]. The research mechanism suggests that bone marrow is the main source of cells for the hematopoietic and immune systems [26]. Both the number of nucleated cells in the bone marrow and the blood cells in the peripheral blood represent the hematopoietic function of the bone marrow [25,27]. Cy could cause reductions in circulating blood cells and in bone marrow cellularity, and at very high doses, it may irreversibly decrease the proliferative potential of hematopoietic stem cells [24]. According to our study, the Cy group showed decreased peripheral blood and BMNC counts. After treatment in the G-LLA group and other drug groups, the mice showed different degrees of recovery of WBCs and BMNCs, and the effect in the G-

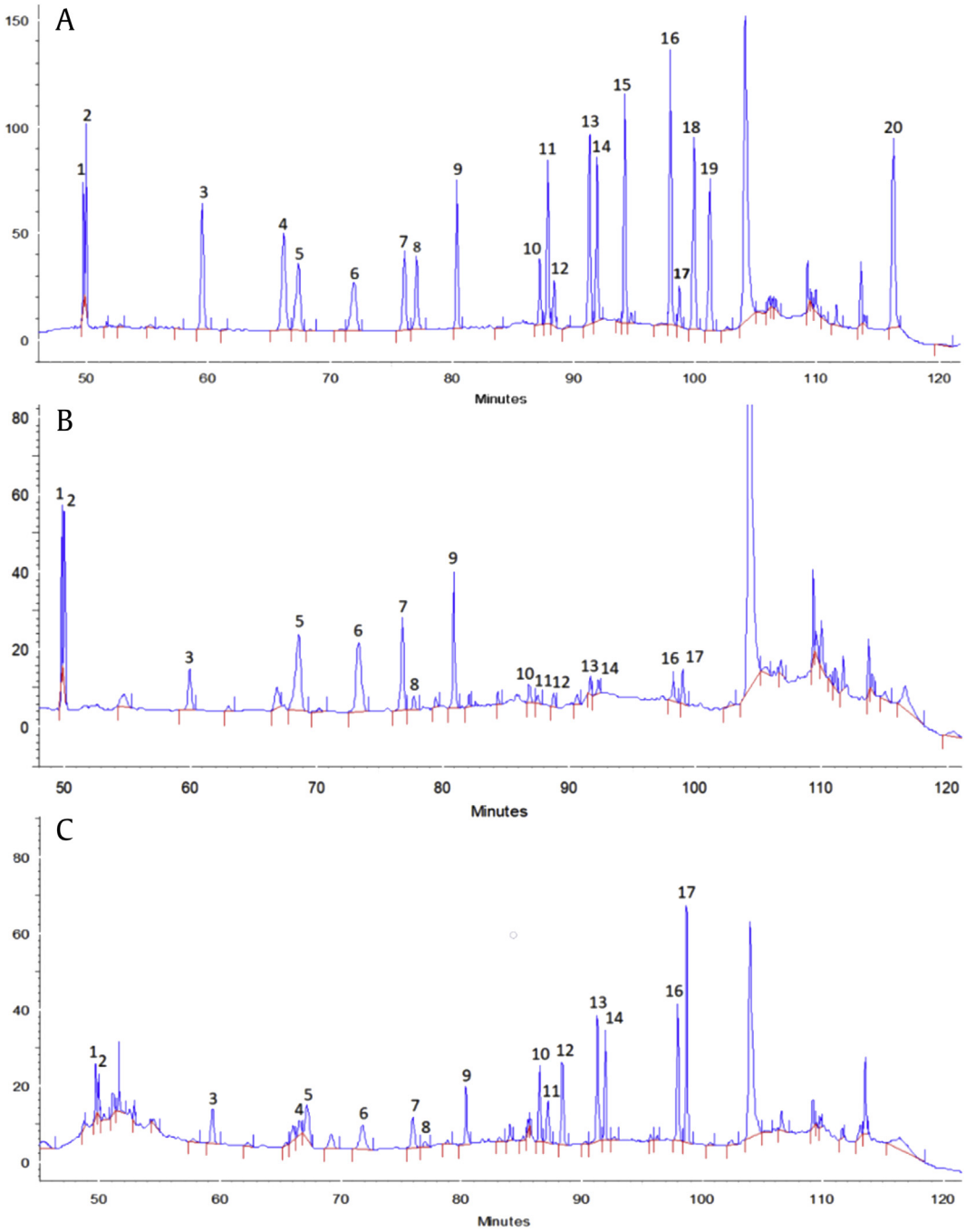

Fig. 5. The remarkable ginsenoside profile differences between G and G-LLA. (A) Calibration curve; (B) G; (C) G-LLA. Peak numbers: 1, Rg_1; 2, Re; 3, Rf; 4, Rg_2; 5, Rb_1; 6, Rc; 7, Rb_2; 8, Rb_3; 9, Rd; 10, Rk_3; 11, F_2; 12, Rh_4; 13, 20(S)-Rg_3; 14, 20(R)-Rg_3; 15, PPT; 16, Rk_1; 17, Rg_5; 18, compound K; 19, Rh_2; 20, PPD. G-LLA, ginseng *Ligustrum lucidum* Ait herb pair.

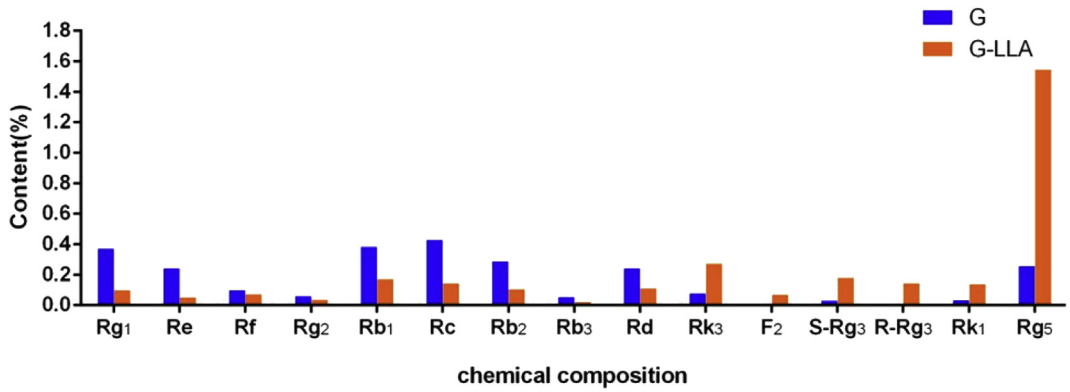

Fig. 6. The remarkable ginsenoside profile differences between G and G-LLA. G-LLA, ginseng *Ligustrum lucidum* Ait herb pair.

LLA group was more obvious, which was not obvious for RBC. This may be explained by the much longer lifespan of RBCs [28].

Thymus and spleen are two main immune organs that play important roles in cellular and humoral immunity. Some scholars advocated that the changes in the thymus index and spleen index can reflect the damage and recovery of the immune system [29]. Thymus and spleen functions can be inhibited by Cy treatment [30]. After treatment with Cy, there was impaired bone marrow hematopoietic function, thymus atrophy, and spleen compensatory enlargement. G-LLA group and other drug groups can have high thymus indices, and G-LLA and G can relieve splenomegaly and normalize the spleen. It suggested that G-LLA was more effective in promoting the recovery of the immune organs of Cy mice.

Hematopoietic progenitors are the product of differentiation of hematopoietic stem cells. Through their proliferation, granulocyte, erythrocyte, and megakaryocyte colonies are formed. The production of these colonies reflects the proliferation of bone marrow hematopoietic cells [31]. After the bone marrow–suppressed mouse model was prepared, G, LLA, and G-LLA were given for 12 days. Then, bone marrow cells were taken out for culture. Experimental results showed that the CFU-GM, BFU-E, CFU-E, and CFU-Meg colony generation rates in the model group were significantly lower than those in the normal group, indicating that the hematopoietic function of the bone marrow was significantly suppressed after model establishment. Compared with the Cy group, G, LLA and G-LLA can promote colony formation of CFU-GM, BFU-E, CFU-E, and CFU-Meg, suggesting that G, LLA, and G-LLA can increase the number of hematopoietic progenitor cells and further promote the recovery of bone marrow hemopoietic function after radiotherapy and chemotherapy injury.

Cytokines emerged as major regulators in health and disease and host-specific response to inflammation, immunity, and cancer [32]. Modern research indicates that hematopoietic cells require the presence and stimulation of specific growth factors for their proliferation and differentiation [33]. GM-CSF is an important growth factor, which can strengthen the production of bone marrow hematopoietic progenitor cells and mobilize them from hematopoietic tissue into blood circulation [34]. And, it has the ability to stimulate the proliferation and maturation of granulocytes and macrophages [35]. TPO, a glycoprotein hormone produced by the liver and kidney, can modulate platelet production [36–38]. EPO is an important regulator of erythropoiesis in mammals and other organisms [39]. Normally, only a small amount of cytokines exists in serum. The amount of cytokines in serum should be increased by Cy. With the treatment of drugs, the amount of cytokines in the microenvironment will gradually fall back to normal. It can be seen from the effect on TPO that all groups can effectively balance the content of TPO. The effect on GM-CSF indicated that all groups had obvious positive regulation effect on GM-CSF and that G-LLA had better regulation effect. It suggested that G-LLA can promote the recovery of cytokines, especially TPO and GM-CSF.

There are two important monitoring points in the cell cycle regulation, located between the G_1 and S phases and the G_2 and M phases [40]. Bone marrow cells in the proliferative phase are most sensitive to chemoradiotherapy injury [41]. After radiochemotherapy, DNA damage is caused in hematopoietic cells, the cell cycle checkpoint mechanism is activated, and the cell cycle is arrested in the G_1 phase to repair damaged DNA. For cells that cannot be repaired, an apoptotic program will be initiated to eliminate damaged cells [42]. The distribution of the various phases of the cell cycle can reflect the degree of damage or recovery of bone marrow cells [43]. In our study, the cell ratio of G_0/G_1 stage increased in the Cy group, whereas the cell ratio of S stage and G_2/M stage decreased significantly. It showed that the DNA damaged cells stagnated in the G_1 phase and led to S phase arrest; G-LLA could promote cells in the bone marrow from G_0/G_1 phase to enter S phase and then the next G_2/M phase. It is suggested that G-LLA could improve cells to pass the G_1/S phase checkpoint and successfully enter the cell cycle to facilitate the repair of damaged DNA and accelerate cell proliferation.

During the processing of ginseng samples, the ginsenosides will be transformed to form rare ginsenosides under the influence of temperature and pH [44]. For ginseng preparations, owing to the complexity of the prescription of TCM preparations, saponins are more likely to undergo structural transformation to form secondary saponins during the preparation process [45,46]. Secondary saponins are rare saponins. Recent scientific studies have shown that rare saponins have better pharmacological effects than conventional saponins in protecting apoptosis of cells [47], inhibition of tumor cells, and immune regulation [48]. According to our study, compatible with G and LLA, the conventional saponins were degraded and transformed into rare ginsenosides. The trialcohol type of saponins Re was transformed into rare saponins Rk_3, and its

Table 4
Chemical contents analysis of LLA and G-LLA

Group	LLA (%)	G-LLA (%)
Salidroside	0.094	0.077
Tyrosol	0.069	0.062
Hydroxytyrosol	0.060	0.062
Ligustroflavone	0.004	0.008
Specnuezhenide	0.281	0.180

G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; LLA, *Ligustrum lucidum* Ait.

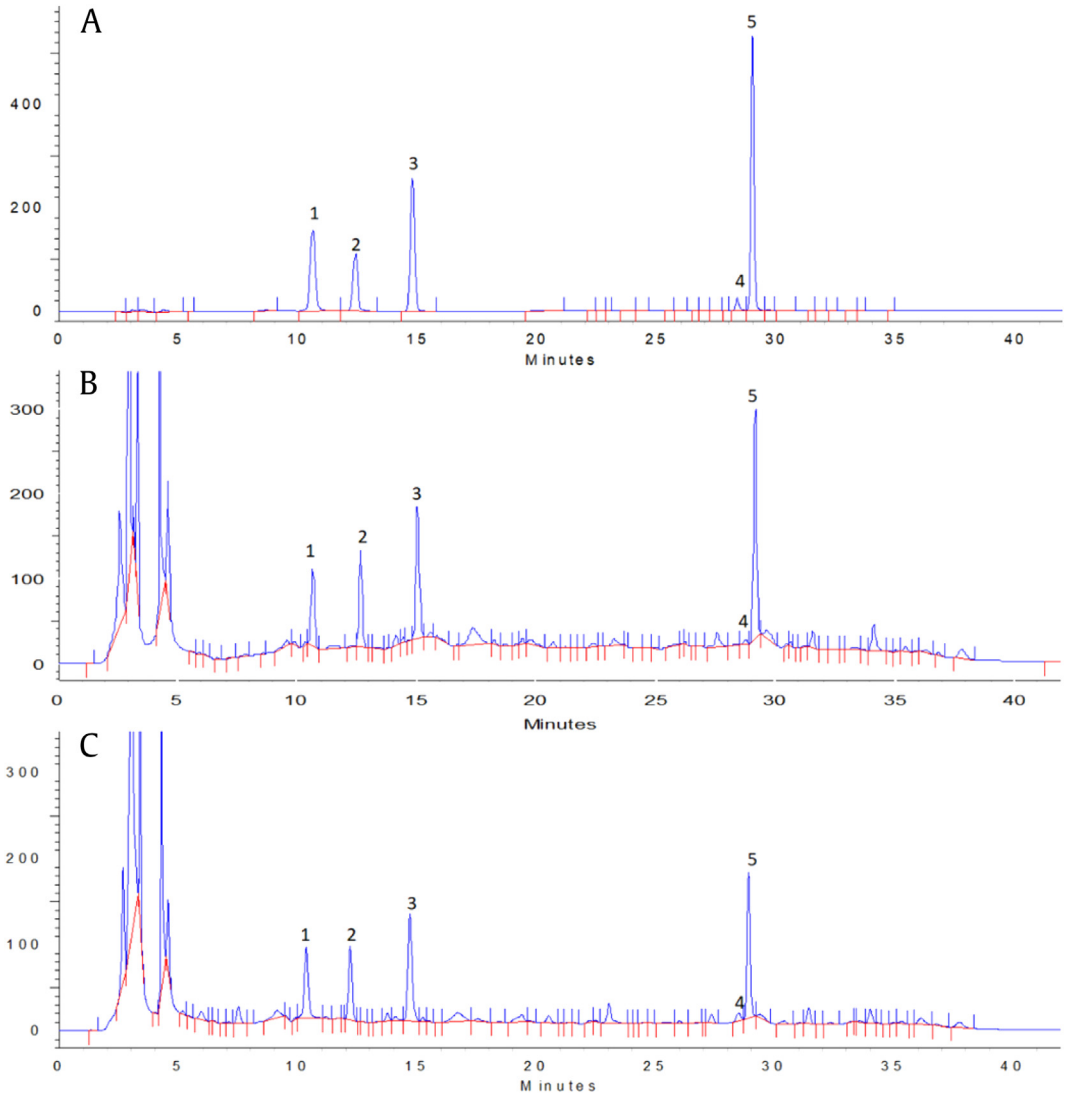

Fig. 7. HPLC chromatograms showing the chemical composition of LLA and G-LLA. (A) Calibration curve; (B) LLA; (C) G-LLA. Peak numbers: 1, hydroxytyrosol; 2, salidroside; 3, tyrosol; 4, ligustroflavone; 5, specnuezhenide. G-LLA, ginseng *Ligustrum lucidum* Ait herb pair.

content was 0.271%. The diol type of saponins Rb_1, Rc, Rb_2, Rb_3, and Rd also underwent transformation to form rare saponins Rg_5, Rk_1, and Rg_3. The content of Rg_5 increased the most, up to 1.544%; the content of Rg_3 is determined out of nothing; and the content of Rk_1 is 0.136%. The production and increase of these rare saponins make G-LLA get more absorbed, which enhances its tonic effect.

The main pharmacologically active substances of Fructus *Ligustrum lucidum* Ait include salidroside, tyrosol, hydroxytyrosol, ligustroflavone, specnuezhenide, and polysaccharide [18,49]. It is reported that salidroside can treat myelosuppression [50]. In our study, although the content of salidroside in G-LLA decreased, it was still very high. Meanwhile, the compatibility of the two drugs increased the dissolution of ligustroflavone. These active ingredients may contribute more to the total pharmacological activity of the herb pairs.

5. Conclusion

In conclusion, the aforementioned results show that G-LLA has ameliorative function against Cy-induced myelosuppression and affects hematopoiesis recovery via improvements of various parameters such as WBC, BMNCs, spleen/thymus index, hematopoietic progenitor, hematopoiesis-related cytokines, and cell cycle. It is suggested that this improvement may be related to the material basis of G-LLA—the change of active ingredients. The findings may be of great importance for the development of G-LLA as a therapeutic agent for the prevention and treatment of human myelosuppression to further study the pharmacodynamic material basis of G-LLA herb pair and provide a new way of thinking for the clinical prescription of TCM.

Conflicts of interest

All the authors declare that there are no conflicts of interest.

Acknowledgments

This work was financially supported by National key R&D program, Key Technology Research of Ginseng Industry and Development of Great Health Products (grant No. 2017YFC1702101), and the national college students innovation and entrepreneurship training

program of Jilin Agricultural University (project No. 201810193044).

References

[1] Zhu JX, Liu R, Jiang ZB, Wang PL, Yao Y, Shen Z. Optimization of drug regimen in chemotherapy based on semi-mechanistic model for myelosuppression. J Biomed Inf 2015;57(C):20–7.
[2] Eichhorst ST, Müerköster S, Weigand MA, Krammer PH. The chemotherapeutic drug 5-fluorouracil induces apoptosis in mouse thymocytes in vivo via activation of the CD95 (APO-1/Fas) system. Cancer Res 2001;61(1):243–8.
[3] Brodsky RA. High-dose cyclophosphamide for autoimmunity and alloimmunity. Immunol Res 2010;47:179–84.
[4] Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology 2000;148(2):187–97.
[5] Henning GT, Schild SE, Stafford SL, Donohue JH, Burch PA, Haddock MG, Gunderson LL. Results of irradiation or chemoirradiation for primary unresectable, locally recurrent, or grossly incomplete resection of gastric adenocarcinoma. Int J Radiat Oncol 2000;46(1):109–18.
[6] Newman NB, Sidhu MK, Baby R, Moss RA, Nissenblatt MJ, Chen T, Lu SE, Jabbour SK. Long-term bone marrow suppression during postoperative chemotherapy in rectal cancer patients after preoperative chemoradiation therapy. Int J Radiat Oncol Biol Phys 2016;94(5):1052–60.
[7] Feng LZ, Huang QJ, Huang ZY, Li H, Qi XX, Wang Y, Liu ZQ, Liu XH, Lu LL. Optimized animal model of CTX-induced bone marrow suppression. Basic Clin Pharmacol Toxicol 2016;119(5):428–35.
[8] Liu XJ, Zhao QL, Sun GX, Williams P, Lu XJ, Cai JZ, Liu WJ. Arsenic speciation in Chinese herbal medicines and human health implication for inorganic arsenic. Environ. Pollut 2013;172(5):149–54.
[9] Kong DX, Li XJ, Zhang HY. Where is the hope for drug discovery? Let history tell the future. Drug Discov Today 2009;14(3):115–9.
[10] Narayanan BA, Narayanan NK, Pttman B, Reddy BS. Adenocarcina of the mouse prostate growth inhibition by celecoxib: downregulation of transcription factors involved in COX-2 inhibition. Prostate 2010;66(3):257–65.
[11] Wang Y, Yan J, Li S, Wang W, Cai X, Huang D, Gong L, Li X. Composition of the essential oil from danggui-zhiqiao herb-pair and its analgesic activity and effect on hemorheology in rats with blood stasis syndrome. Pharmacog Mag 2016;12(48):271–5.
[12] Zhao XN, Li YR, Huai JX, Cheng CC, Zhang T, Xie LL, Wang SQ, Zhang M, Dai RH. Compatibility effects of herb pair Phellodendri chinensis cortex and Anemarrhenae rhizoma on benign prostatic hyperplasia using targeted metabolomics. Biomed Chromatogr 2018.
[13] Mei F. Study on chemical constituents, pharmacodynamics effects and metabolomics of traditional Chinese hreb pair, ephedra and gypsum. Southern medical university; 2013.
[14] Yuan HD, Kim JT, Kim SH, Chung SH. Ginseng and diabetes: the evidences from in vitro, animal and human studies. J. Ginseng Res 2012;36(1):27–39.
[15] Shin JY, Lee JM, Shin HS, Park SY, Yang JE, Cho SK, Yi TH. Anticancer effect of ginsenoside F2 against glioblastoma multiforme in xenograft model in SD rats. J. Ginseng Res 2012;36(1):86–92.
[16] Kim JH. Cardiovascular diseases and Panax ginseng: a review on molecular mechanisms and medical applications. J. Ginseng Res 2012;36(1):16–26.
[17] Ernst E. Panax ginseng: an overview of the clinical evidence. J. Ginseng Res 2010;34(1):259–63.
[18] Liu TT, Wang M. Research progress of chemical composition and pharmacological effects of fructus figustri lucidi. Chin J Exp Tradit Med Formulae 2014;20(14):28–234.
[19] Wang ZX, Gao BZ, Xu BY, Huang GC. Study on anti - mutagenicity of Fructus Ligustri Lucidi in fruit fly test. Fujian J Tradit Chin Med 1991;22(3):50–1.
[20] Chen ZW, Xu HY, Zhang X, Wu N, Ju Y, Han L, Yu W. Effect of formlula granule of Siwu on LN, FN, FAK of BMSC in bone marrow depression mice. China Occup Med 2009;36(3):205–7.
[21] Wei T, Zou SP, Liu QP, Chen W. The treatment of 45 cases of leukopenia after chemotherapy with huoxue shengbai decoction. Shaanxi J Tradit Chin Med 2009;30(6). 698–698.
[22] Zeng BR, He X. Clinical observation of anti - tumor shengbai tablet in preventing bone marrow injury by radiotherapy and chemotherapy. J Tradit Chin Med Univ Hunan 2007;27(2):71–2.
[23] Kaina B, Christmann M, Naumann S, Roos WP. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. DNA Repair (Amst) 2007;6(8):1079–99.
[24] Kaufmann SH, Peereboom D, Buckwalter CA, Svingen PA, Grochow LB, Donehower RC, Rowinsky EK. Cytotoxic effects of topotecan combined with various anticancer agents in human cancer cell lines. J Natl Cancer Inst 1996;88(11):734–41.
[25] Xu SF, Yang TH, Wu Q, Fan Z, Zhang W, Yu LM. Restorative effect of Astragalus Polysaccharides on Hematopoietic Function in cyclophosphamide-induced bone marrow depression in mice. Acta Academiae Medicinae Zunyi 2011;34(5). 473–473.
[26] Zhao L, Wang Y, Shen HL, Shen XD, Nie Y, Wang Y, Han T, Yin M, Zhang QY. Structural characterization and radioprotection of bone marrow hematopoiesis of two novel polysaccharides from the roof of Angelica sinensis (Oliv.) Diel. Fitoterapia 2012;83(8):1712–20.
[27] Qiu RF, Wang YH, Gao Q, Li S, Lu ZH. Effects of glycosaminoglycan from Apostichopus japonius on peripheral blood cells and bone marrow cell cycle of myelosuppressed anemia mice. Chinese J Mar Drugs 2015;34(1):47–52.
[28] Sun X, Zhao YN, Qian S, Gao RL, Yin LM, Wang LP, Chong BH, Zhang SZ. Ginseng-derived panaxadiol saponins promote hematopoiesis recovery in cyclophosphamide-induced myelosuppressive mice: potential novel treatment of chemotherapy-induced cytopenias. Chin J Integr Med 2018;(3):1–7.
[29] Huang GH, Deng H, Fu X. Effects of ethanol extract from elderflower on glucose tolerance and thymus and spleen index in diabetic mice. Chin J Exp Tradit Med Formulae 2012;18:183–6.
[30] Zhao N, Wang L, Mou HY, Liang M, Yue W. Synergism and attenuation effects of taurine on cyclophosphamide. Chin J Cancer 2009;28(3):244–8.
[31] Xiao WC. Comparison of effects of liuwei dihuang pill and guifu dihuang pill on hematopoiesis in mice induced by radiotherapy and chemotherapy. Chengdu university of Traditional Chinese Medicine; 2009.
[32] Dinarello CA. Proinflammatory cytokines. Chest 2000;118(2):503–8.
[33] Lu Y, Feng XM, Zhu BD. Effects of total saponin of panax ginseng on Epo/EpoR in model mice with myelosuppression. Space Med Med Eng 2005;18(5):384–6.
[34] Ma ZC, Hong Q, Wang YG, Tan HL, Xiao CR, Liang QD, Lu BB, Gao Y. Effects of ferulic acid on hematopoietic cell recovery in whole-body Gamma irradiated mice. Int J Radiat Biol 2011;87(5):499–505.
[35] Metcalf D. The granulocyte–macrophage colony-stimulating factors. Science 1985;229:16–22.
[36] Zhao AB, Yu B, Wu XL, Cao KJ, Li EQ, Li QM, Chen XY. Protective effects on myelosuppression mice treated by three different classic Chinese medicine formulae. Pharmacogn Mag 2011;7(26):133–40.
[37] Wagemaker G, Neelis KJ, Hartong SCC, Wognum AW, Thomas GR, Fielder PJ, Eaton DL. The efficacy of recombinant TPO in murine and nonhuman primate models for myelosuppression and stem cell transplantation. Stem Cells 1998;16(Suppl 2):127–41.
[38] Abina MA, Tulliez M, Lacout C, Debili N, Villeval JL, Pflumio F, Wendling F, Vainchenker W, Haddada H. Major effects of TPO delivered by a single injection of a recombinant adenovirus on prevention of septicemia and anemia associated with myelosuppression in mice: risk of sustained expression inducing myelofibrosis due to immunosuppression. Gene Ther 1998;5(4):497–506.
[39] Ma ZC, Hong Q, Wang YG, Tan HL, Xiao CR, Liang QD, Wang DG, Gao Y. Ferulic acid protects lymphocytes from radiation-predisposed oxidative stress through extracellular regulated kinase. Int J Radiat Biol 2011;87(2):130–40.
[40] Israels ED, Israels LG. The cell cycle. Stem Cell 2001;19:88–91.
[41] Zheng YF, Jiang JQ, Zhang LH, Shi YP, Huang Q. The effect of you gui wan on bone marrow cell cycle and apoptosis of Myelosuppression mice. J Mil Surgeonin Southwest China 2009;11(3):395–7.
[42] Zhan QM. Molecular oncology. Beijing: people's medical publishing house; 2005. p. 322–6.
[43] Zhao JH, Zhu BD, Huang Q, Wang P, Qi GH. Effects of Shengyu decoction on bone marrow,cell cycle and apoptosis of myelosuppressed mice. Chin J Exp Tradit Med Formulae 2011;17(16):119–202.
[44] Zhang WY, Chen QC, Zheng YN, Wang JY, Li FW, Wang DH, Liu LN. Determination of ginsenoside Compound K in different parts of Panax ginseng by RP-HPLC. Chin J Pharmaceut Anal 2007;27(1):1–3.
[45] Hua GD, Gong Y, Liu WY, He T, Zhao W. Effects of different processing methods on ginsenosides and pesticide residues. Beijing Tradit Chin Med 2014;33(8):627–31.
[46] Wan JY, Fan Y, Yu QT, Ge YZ, Yan CP, Alolga RN, Li P, Ma ZH, Qi LW. Integrated evaluation of malonyl ginsenosides, amino acids and polysaccharides in fresh and processed ginseng. J Pharmaceut Biomed Anal 2015;107:89–97.
[47] Kim HY, Kim K. Protective effect of ginseng on cytokineinduced apoptosis in pancreatic β-cells. J Agric Food Chem 2007;55(8):2816–23.
[48] Chen F, Sun Y, Zheng SL, Qin Y, McClements DJ, Hu JN, Deng ZY. Antitumor and immunomodulatory effects of ginsenoside Rh2 and its octyl ester derivative in H22 tumor-bearing mice. J Funct Foods 2017;32:382–90.
[49] Cheng M, Hu ZH. Advances in the study of the biological and chemical components of Fructus Ligustri Lucidi. Chin Tradit Herbal Drugs 2010;41(7):1219.
[50] Wu JL, Wang PH, Wang X, Yang SX. Effects of salidroside on hematopoietic recovery in cyclophosphamide-induced marrow injury mice. Tradit Chin Drug Res Clin Pharmacol 2013;24(4):371–4.