Original Research

Protective effect of Dammarane Sapogenins against chemotherapy-induced myelosuppression in mice

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Abstract

Chemotherapy is the most common way to treat malignancies, but myelosuppression, one of its common side-effects, is a formidable problem. The present study described the protective role of Dammarane Sapogenins (DS), an active fraction from oriental ginseng, on myelosuppression induced by cyclophosphamide (CP) in mice. DS was orally administered at different dosages (37.5, 75, and 150 mg/kg) for 10 d after CP administration (200 mg/kg intraperitoneally). The results showed that DS increased the number of white blood cells (WBC) on day 3 and day 7 ($P < 0.05$), such that WBC levels were increased by 105.7 ± 29.5% at 75 mg/kg of DS on day 3 ($P < 0.05$, compared with the CP group). Similar results were observed in red blood cells and platelets in DS-treated groups. The colony-forming assay demonstrated that the depressed numbers of CFU-GM (colony-forming unit-granulocyte and macrophage), CFU-E (colony-forming unit-erythroid), BFU-E (burst-forming unit-erythroid), CFU-Meg (colony-forming unit-megakaryocyte) and CFU-GEMM (colony-forming unit-granulocyte, -erythrocyte, -monocyte and -megakaryocyte) induced by CP were significantly reversed after DS treatment. Moreover, the ameliorative effect of DS on myelosuppression was also observed in the femur by hematoxylin/eosin staining. In DS-treated groups, ConA-induced splenocyte proliferation was enhanced significantly at all the doses (37.5, 75, 150 mg/kg) on day 3 at the rate of 50.3 ± 8.0%, 77.6 ± 8.5% and 44.5 ± 8.4%, respectively, while lipopolysaccharide-induced proliferation was increased mainly on day 7 ($P < 0.01$), with an increased rate of 39.8 ± 5.6%, 34.9 ± 6.6% and 38.3 ± 7.3%, respectively. The thymus index was also markedly increased by 70.4% and 36.6% at 75 mg/kg on days 3 and 7, respectively, as compared with the CP group. In summary, DS has a protective function against CP-induced myelosuppression. Its mechanism might be related to stimulating hematopoiesis recovery, as well as enhancing the immunological function.

Keywords: Dammarane Sapogenins, cyclophosphamide, myelosuppression, hematopoiesis, immunoregulation, splenocyte proliferation

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Introduction

Chemotherapy is one of the most widely used methods in cancer treatments. However, anti-cancer reagents are usually toxic to normal cells and organs, consequently causing undesirable side-effects such as nausea, vomiting, alopecia, myelosuppression and renal toxicity.[1,2] Of these side-effects, myelosuppression is the most common, and it not only affects the chemotherapy regimen but also dramatically decreases the quality of the patient's life. Clinically, granulocyte colony-stimulating factors (G-CSFs) and other colony-stimulating factors (CSFs)[3,4] are usually used to alleviate myelosuppression induced by chemotherapy. However, their application is limited due to the severe side-effects,[5–7] such as bone pain and thrombocytopenia. Thus, there is a dire need for developing new chemoprotective agents with low toxicities.

It has been reported earlier that total ginsenosides derived from Radix Ginseng and other species in the ginseng family (Araliaceous plants) possess various biological activities, including immunomodulatory,[8] antitumor,[9] antidementia,[10]

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antiaging[11,12] and many other pharmacological actions.[13,14] Among the total ginsenosides, Rb1 and Rg1 are regarded as the main active components.[15] Their pharmacological actions have been attributed to the deglycosylated aglycone metabolites formed in the intestine[15,16] having greater biological effects than the ginsenosides.[17,18] The intestinal metabolites of Rb1 and Rg1 are PPD (20(S)-aglycone protopanaxadiol) and PPT (20(S)-aglycone protopanaxatriol), respectively (Figure 1), which are easily absorbed and exhibit more potent effects.[19–22] Until now, the focus has been on the anticancer activity of the metabolites of ginsenosides;[23,24] however, their effects against myelosuppression have not been addressed. This paper focuses on investigating the effects of Dammarane Sapogenins (DS) on cyclophosphamide (CP)-induced myelosuppression in mice and their possible mechanism of action.

Materials and methods

DS preparation

DS was provided by Pegasus Pharmaceuticals Group Inc. (Richmond, BC, Canada). DS, a proprietary powdered Asian ginseng extract exclusively available at Pegasus Pharmaceuticals Group Inc. (USA Patent No. US 6,888 014B2, May 3, 2005, Huang D and Qi DF) was prepared by alkaline hydrolysis of total ginsenosides derived from the stem and leaf of *Panax ginseng*. Briefly, the total ginsenoside powder was processed with a sodium hydroxide/water solution at elevated temperature (150–300°C) and pressure (2.5–8.4 MPa) for approximately 1.5 h in a 50 L tank. Upon the completion of the procedure, the liquid content was separated from the solid content that contains a hydrolyzed ginsenoside, and neutralized with hydrochloric acid prior to disposal. The hydrolyzed ginsenoside was further purified by ethanol extraction, followed by filtration, ethanol/water precipitation, and spin-drying. Subsequently, this spin-dried solid, the refined DS, was placed into an oven for drying under vacuum.

The HPLC system (Thermo Electron, San Jose, CA, USA) used for further quantification was equipped with a 126 solvent delivery module, a 168 photo diode-array detector and a 507e autosampler. A 5 μL aliquot of sample was injected into a Diamonsil C18 column (5 μm, 4.6 × 150 mm, maintained at 35°C) eluted with a mobile phase consisting of water and acetonitrile (38:62). The effluent was monitored at 203 nm (diode array detector). The refined DS contains 33% PPT and 16% PPD on the anhydrous basis (Figure 2).

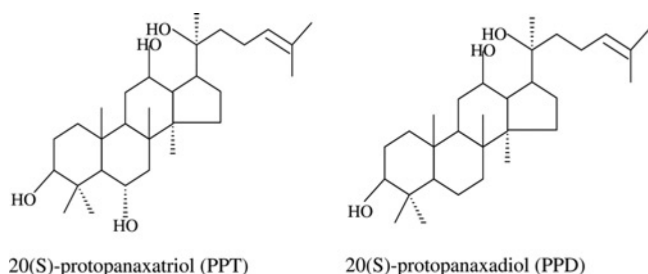

Figure 1 Structure of the main ingredients in Dammarane Sapogenins

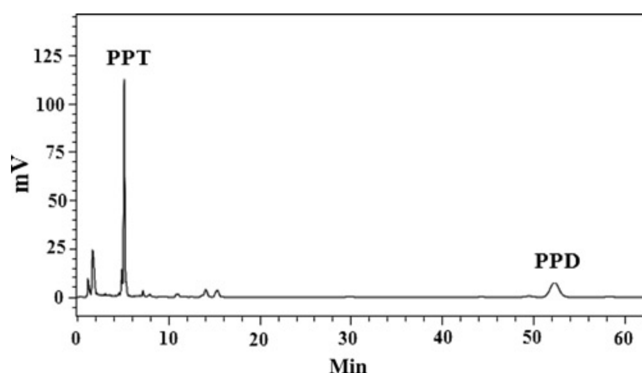

Figure 2 Typical high-performance liquid chromatography chromatogram of Dammarane Sapogenins
PPT, 20(s)-protopanaxatriol; PPD, 20(s)-protopanaxadiol

Chemicals agents

Methylcellulose medium M3534 and M3334 were purchased from Stem Cell Technology (Vancouver, BC, Canada), and RPMI-1640 medium and fetal bovine serum were obtained from Gibco, Life Technologies Inc. (Paisley, UK). CP was obtained from Jiangsu Hengrui Medicine Co., Ltd (Beijing, China). All the other reagents and drugs were obtained from Sigma (St Louis, MO, USA).

Animal grouping and treatment

Male BALB/c mice (6–8 weeks) were supplied by the Institute of Laboratory Animal Center, Chinese Academy of Military Medical Sciences, Beijing, China. They were housed in an air-conditioned SPF room (temperature: 22 ± 2°C) with a 12 h light/dark cycle, and fed with commercial standard rodent diet and water *ad libitum*.

After three days of acclimatization, mice were divided into five groups. Mice in the CP and DS groups were injected intraperitoneally with a single dose of CP (200 mg/kg, 0.2 mL/10 g) on day 0, and the control group received an equal volume of saline correspondingly. Immediately after CP treatment, mice (of the DS group) were orally administrated three dosages of DS (37.5, 75 and 150 mg/kg, 0.2 mL/10 g). The control and CP groups received an equivalent volume of distilled water, once a day for 10 consecutive days.

All procedures involving animals and their maintenance were conducted in compliance with the Guide of the Care and Use of Laboratory Animals (NIH publication No. 86–23, revised 1996) after approval by the Animal Ethics Committee at Peking Union Medical College (Beijing, China).

Isolation of mouse bone marrow cells

On day 7, animals were sacrificed by anesthesia. The femurs were dissected, collected and flushed with 3 mL of RPMI-1640 medium containing fetal bovine serum (2%) to obtain bone marrow cells (BMCs) which were pooled from each group ($n = 3$). To a sample of cell suspension (20 μL), acetic acid solution (180 μL, 3%) was added for red blood cell (RBC) lysis, mixed with 0.04% trypan blue and

nucleated cells were counted under light microscope. The cell concentration was adjusted to 1×10^6 cells/mL with RPMI medium and kept on ice until the colony-forming cells (CFC) assay.

Mouse CFC assay

Cell suspension (100 μL) was added to 900 μL of methylcellulose medium M3534 containing recombinant murine interleukin (IL)-3 (rmIL-3, 10 ng/mL), human IL-6 (rhIL-6, 10 ng/mL), mouse stem cell factor (rmSCF, 50 ng/mL) or M3334 containing recombinant murine erythropoietin (rhEPO, 3 U/mL). The culture system of CFU-Meg (colony-forming unit-megakaryocyte) and CFU-GEMM (colony-forming unit-granulocyte, -erythrocyte, -monocyte and -megakaryocyte) was composed of horse serum (25%,) erythropoietin (2 U/mL), glutamine (0.045%), rmSCF (20 ng/mL), IL-11 (10 ng/mL), 2-mercaptoethanol (0.01 mmol/L), IL-3 (10 ng/mL), methylcellulose (1%) and nucleated cells (5×10^5/mL). The above mixture (1 mL) was placed into 12-well plates and incubated at 37°C in a humidified atmosphere of 5% CO_2. CFU-E (colony-forming unit-erythroid) were counted after three days, and CFU-GM (colony-forming unit-granulocyte and macrophage), BFU-E (burst-forming unit-erythroid), CFU-Meg and CFU-GEMM after seven days. The results are expressed as colonies/2×10^4 or 1×10^5 nucleated cells.

Hematological examination and histological examination of bone marrow

On days 3 and 7, blood samples (20 μL) were collected from the tail vein and white blood cells (WBC), RBC and platelets (PLT) were measured by a cell counter (Sysmex 800, Sysmex, Kobe, Japan). On day 3, after the mice were sacrificed, the femurs were fixed in paraformaldehyde (4%) in phosphate-buffered saline (PBS) overnight, decalcified and embedded. The 4-μm sections were prepared and stained with hematoxylin/eosin[25] and photographed.

Determination of spleen and thymus indices

On days 3 and 7, animals were sacrificed; spleens and thymus were removed and weighted. The spleen/thymus index was calculated as the weight of the spleen or thymus (mg)/10 g body weight.

Analysis of splenocyte proliferation

On days 3 and 7, spleens were aseptically removed from mice, and the cell suspensions were passed through fine nylon mesh, followed by centrifugation (1500 rpm for 5 min) and washed twice in PBS. After RBC lysis (3% acetic acid) as described above, the cell suspension was adjusted to 5×10^6 cells/mL with RPMI-1640 medium containing fetal bovine serum (10%). Splenocytes (100 μL) were seeded into 96-well plates in the presence of either RPMI-1640 or mitogens, i.e. lipopolysaccharide (LPS) (10 μg/mL) or ConA (5 μg/mL) and incubated for 48 h at 37°C. The proliferation was measured using 3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.[26] The optical density was determined using a microplate ELISA reader at 570 nm (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). The stimulation index stimulated by ConA (CSI)/LPS (LSI)/was calculated as follows:

$$SI = OD_{(\text{mitogen-stimulated cultures})} - OD_{(\text{medium})} / OD_{(\text{non stimulated cultures})} - OD_{(\text{medium})}$$

Statistical analysis

Data were analyzed by one-way analysis of variance followed by least significant difference or Dunnett's test. Histograms were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The data were presented as mean ± standard error (SE) and $P \leq 0.05$ was considered statistically significant.

Results

Effects of DS on peripheral hemogram

Table 1 shows that administration of CP (200 mg/kg) caused a significant (86.0 ± 0.7%) decline in WBC (from 14.82×10^6 down to 2.07×10^6/mL) on day 3 and a 35.5 ± 7.8% decline on day 7 (from 15.89×10^6 down to 10.26×10^6/mL; $P < 0.01$, compared with the control group). The DS antagonized the decline in WBC induced by CP, as WBC levels decreased by only 69.3% at 75 mg/kg of DS on day 3, while the levels in the CP group decreased significantly by 86.0% ($P < 0.05$). In addition, there was about 20% reduction at both 37.5 and 150 mg/kg of DS on day 7, as compared with 35.5% in the CP group ($P < 0.05$).

Table 1 Effects of DS on the numbers of circulating WBC, RBC and PLT in CP-induced myelosuppressed mice ($n = 12$, means ± SE)

	WBC count (×10^6/mL)		RBC count (×10^9/mL)		PLT count (×10^6/mL)	
Groups	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
Control	14.82 ± 0.85	15.89 ± 0.71	10.98 ± 0.55	10.83 ± 0.40	1265.00 ± 40.26	1150.40 ± 36.79
CP (200 mg/kg)	2.07 ± 0.15##	10.26 ± 0.61##	11.75 ± 0.44	8.91 ± 0.20##	1123.42 ± 43.68	662.85 ± 35.86##
CP + DS						
37.5 mg/kg	3.13 ± 0.16	12.70 ± 0.60*	11.58 ± 0.27	10.18 ± 0.21**	1116.64 ± 73.39	804.38 ± 52.04*
75 mg/kg	4.55 ± 0.69*	11.82 ± 0.60	11.34 ± 0.38	9.59 ± 0.25	1145.76 ± 36.66	816.00 ± 40.51*
150 mg/kg	3.85 ± 0.66	12.68 ± 1.05*	11.92 ± 0.49	9.78 ± 0.34*	1222.00 ± 75.67	682.17 ± 56.62

WBC, white blood cells; RBC, red blood cells; PLT, platelets; CP, cyclophosphamide; DS, Dammarane Sapogenins
#$P < 0.05$, ##$P < 0.01$ compared with control group. *$P < 0.05$, **$P < 0.01$ compared with the CP group

Table 1 also indicates that the numbers of RBC decreased by 17.7 ± 3.1% in the CP group on day 7, while a low but significant effect (decreased by only 6 – 11.5%, as compared with 17.7% for the CP-only-treated cells) was observed after DS treatment ($P < 0.01$, as compared with the CP group). The decline of PLT induced by CP also was significantly reversed by DS on day 7 at 37.5 and 75 mg/kg (about 30% at both dosages as compared with 42.4% for CP group). No significant effect was observed at the higher concentration (150 mg/kg) of DS.

Effects of DS on mouse CFC

As shown in Figures 3a–e, CP (200 mg/kg) significantly reduced the colony numbers in the bone marrow (CFU-GM: 63.2 ± 5.6%, CFU-E: 73.7 ± 6.5%, BFU-E: 71.6 ± 6.8%, CFU-Meg: 50.2 ± 3.4% and CFU-GEMM: 51.6 ± 3.7%, respectively) as compared with the control group. After treatment with DS, the numbers of CFU-GM, CFU-E, BFU-E, CFU-Meg and CFU-GEMM were markedly increased ($P < 0.05$ or 0.01, compared with the CP group). The maximum amount of CFU-GM was generated at the dose of 75 mg/kg and that of other colonies (CFU-E, BFU-E, CFU-Meg and CFU-GEMM) at the 150 mg/kg dose of DS.

Effects of DS on the histopathology of bone marrow

The effect of DS on the histopathology of bone marrow is shown in Figure 4. All the bone marrow cells in the control group contain clusters of hematopoietic cells admixed with adipocytes, erythroid and myeloid cells (Figure 4a). Three days after administration of CP, the number of nucleated myelocytes (blue) in the bone marrow was sharply reduced (by 93.6% compared with the control group) and replaced by vacuolation (Figure 4b). After treatment of DS, the cellular density in the bone marrow clearly increased (by 46.6–81.0% compared with the CP group) with abundant hematopoietic cells (Figures 4c–e). The histopathology of bone marrow did not show a significant difference among all groups on day 7 (data not shown).

Effects of DS on the spleen/thymus index of CP-induced myelosuppressed mice

In the control group, the spleen index was 44.4 ± 0.2 on day 3, and 41.7 ± 0.3 on day 7. It was reduced (by 52.9%) in the presence of CP on day 3, while it was increased (by 80.3%) on day 7 compared with the control group ($P < 0.01$). However, DS had no effect on the spleen index (Table 2). Administration of CP induced a significant decline (by about 60%) of the thymus index on days 3 and 7 compared with the control. Following DS administration, there were 70.4% and 36.6% increase in thymus index at 75 mg/kg on days 3 and 7 respectively, as compared with the CP group.

Effects of DS on splenocyte proliferation

Splenocyte proliferation stimulated by ConA (CSI) was decreased in the CP-treated animals on days 3 and 7 (by 28.8–55.5%, as compared with the control group, see Figure 5). Compared with the CP group, DS reversed ConA-induced proliferation on day 3 at all three dosages (44.5 to 77.6%, $P < 0.01$), but had no effect on CSI on day 7. CP treatment also inhibited LPS-induced splenocyte proliferation (LSI) on days 3 and 7. While only the lower dose of DS (37.5 mg/kg) elicited an increasing effect (15.2 ± 1.1%) on the stimulation index on day 3, all three DS dosages stimulated LPS-induced proliferation of splenocytes on day 7 (by 39.8 ± 5.6%, by 34.9 ± 6.6% and by 38.3 ± 7.3%, respectively) compared with the CP group.

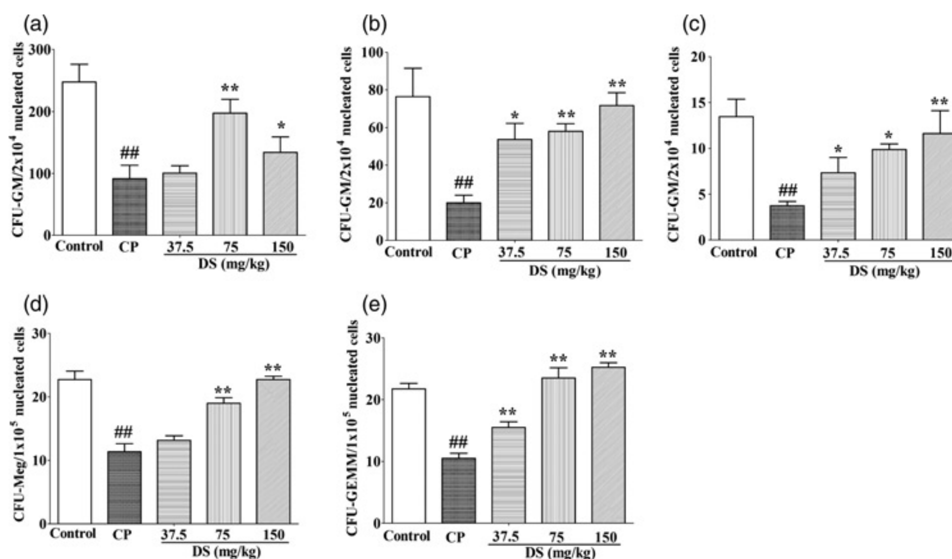

Figure 3 Effects of DS on the numbers of CFU-GM (a), CFU-E (b), BFU-E (c), CFU-Meg (d) and CFU-GEMM (e) in the bone marrow on day 7 after CP treatment. Values represent mean ± SE ($n = 6$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the CP group. CFU-GM, colony-forming unit-granulocyte and macrophage; CFU-E, colony-forming unit-erythroid; BFU-E, burst-forming unit-erythroid; CFU-Meg, colony-forming unit-megakaryocyte; CFU-GEMM, colony-forming unit-granulocyte, -erythrocyte, -monocyte and -megakaryocyte; CP, cyclophosphamide; DS, Dammarane Sapogenins

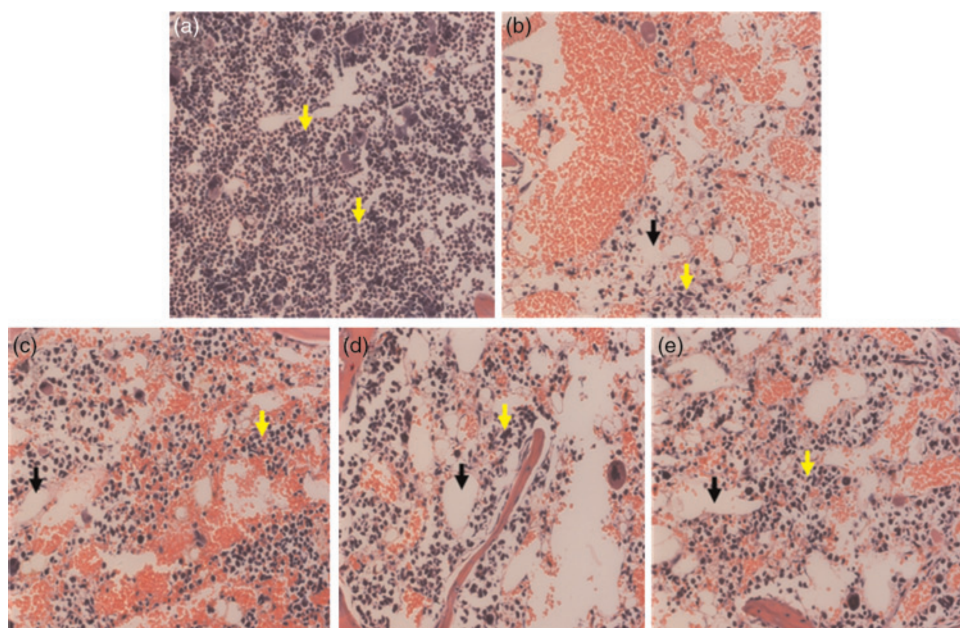

Figure 4 Pathological changes in the bone marrow of mice on day 3. (a) Control, (b) CP-treated (200 mg/kg) and CP + different doses of DS, (c) 37.5 mg/kg, (d) 75 mg/kg and (e) 150 mg/kg. Yellow arrows point to the nucleated cells and black arrows point to vacuolation (magnification × 200). CP, cyclophosphamide; DS, Dammarane Sapogenins (A color version of this figure is available in the online journal)

Discussion

The animal model of CP-induced myelosuppression has been well documented in the evaluation of the hemopoietic activity of cytoprotective agents, such as recombinant human G-CSF or murine G-CSF and amifostine.[27–29] In the present study, CP was also used to induce myelosuppression. Clinical hematopoietic parameters such as WBC, RBC and PLT are routinely used to accurately monitor the chemotherapeutic adverse effects.[30] In our study, after CP treatment (data not shown), peripheral blood WBC numbers declined from day 1 and the decrease in PLT and RBC were observed on day 7, which may be owing to their longer lifespan than WBC in the circulation.[31] DS administration induced a marked increase in WBC, RBC and PLT, which might facilitate the recovery of pancytopenia.

To further verify the above results, colony-forming assays were performed to evaluate the proliferation of hemopoietic progenitor, which is regarded as the premise of peripheral blood cell recovery after bone marrow suppression. CFU-E and BFU-E represent the proliferative capacity of erythroid progenitors, whereas CFU-GM represents granulocyte/macrophage progenitor cells and CFU-Meg is responsible for the production of platelets.[32] Our results indicated that the hemopoietic progenitors in the bone marrow were also significantly enhanced by DS (37.5–150 mg/kg). In addition, the colony size in DS groups was larger than the CP group. These results favored earlier findings on other active ingredients (polysaccharide and Rg1) extracted from ginseng.[33,34] Additionally, histopathological analysis of bone marrow suggested that DS administration not only improved the density of bone marrow cells but the number of nucleated cells as well, which further supports its protective role on hematopoiesis.[35]

In addition to myelosuppression, CP also inhibits the proliferation of immunocytes in the bone marrow, which subsequently results in immunosuppression with the defect in both B and T lymphocytes.[36,37] T- and B-lymphocyte immunity can be detected by Con A- and LPS-induced lymphocyte proliferation assay, respectively.[38,39] Our results indicated that DS significantly augmented Con A-stimulated T-lymphocyte proliferation on day 3 and enhanced LPS-stimulated B-lymphocyte proliferation on day 7. T-lymphocytes are responsible for cell-mediated immunity and B-lymphocytes mediate humoral immunity[40] and their proliferation reflects the functional status of immunocytes. Our results suggest that DS enhanced cell-mediated immunity on day 3 and humoral immunity on day 7 in CP-myelosuppressed mice. However, why DS facilitates different types of lymphocyte proliferation at different times needs to be further elucidated.

Thymus and spleen are important immune organs, and thymus index and spleen index reflect the immune function of an organism.[41] DS significantly restored the thymus

Table 2 Effects of DS on the spleen/thymus index of CP-induced myelosuppressed mice

Groups	Spleen index		Thymus index	
	Day 3	Day 7	Day 3	Day 7
Control	44.4 ± 0.2	41.7 ± 0.3	20.5 ± 0.2	19.6 ± 0.2
CP (200 mg/kg)	20.9 ± 0.2##	75.2 ± 1.6##	8.1 ± 0.1##	8.2 ± 0.1##
CP + DS				
37.5 mg/kg	21.0 ± 0.2	64.2 ± 1.3	9.5 ± 0.1	10.3 ± 0.2
75 mg/kg	20.7 ± 0.2	73.9 ± 0.7	13.8 ± 0.2**	11.2 ± 0.1*
150 mg/kg	20.2 ± 0.3	70.2 ± 1.3	12.1 ± 0.1*	11.1 ± 0.1*

CP, cyclophosphamide; DS, Dammarane Sapogenins
Data represent as the mean ± SE, $n = 6$
#$P < 0.05$, ##$P < 0.01$ versus control group. *$P < 0.05$, **$P < 0.01$ versus CP group

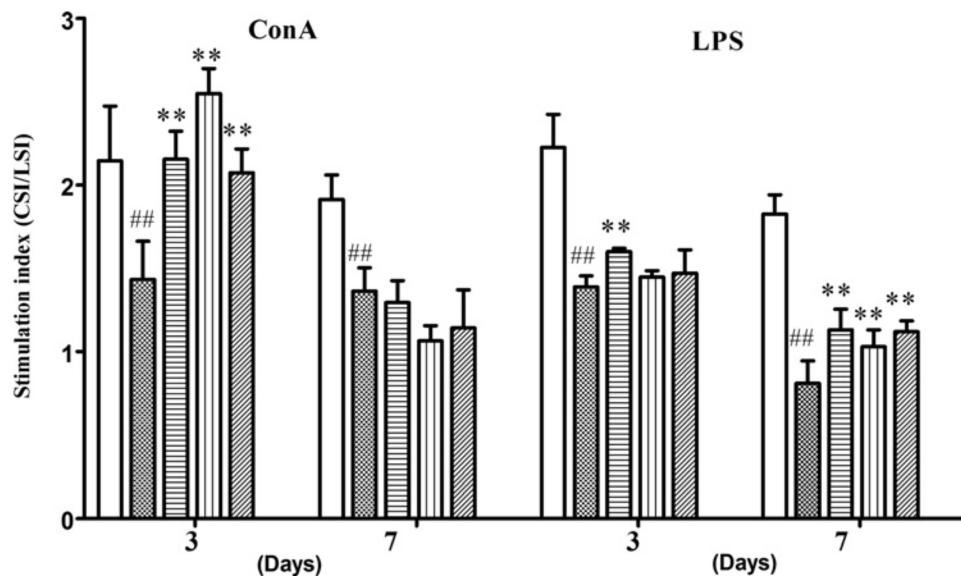

Figure 5 Effects of DS treatment on ConA and LPS-induced proliferation of splenocytes in myelosuppressed mice. Control (▭), CP group (▩) and CP + DS (37.5,▤; 75,▥; 150 mg/kg▨). Splenocytes (5×10^6 cells/mL) were stimulated with either ConA (5 μg/mL) or LPS (10 μg/mL) for 48 h. The value is presented as mean ± SE ($n = 6$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control group and $^{*}P < 0.05$, $^{**}P < 0.01$ versus CP group. CP, cyclophosphamide; DS, Dammarane Sapogenins; LPS, lipopolysaccharide

index decreased by CP but failed to display any detectable effect on the spleen index in our experiment. The spleen index was detected both on day 3 and day 7; the results showed that CP caused marked atrophy of the spleen on day 3 and swelling on day 7. It is probable that changes in the spleen masked the effect of DS; therefore no difference in the spleen index was observed.

Meanwhile, acute lethal toxicity test suggested that DS was non-toxic and oral administration of DS (8 g/kg) had no obvious toxicity to mice (data not shown).

In conclusion, DS has a protective function against CP-induced myelosuppression and affects immunoregulation and hematopoiesis recovery via enhancement of various parameters such as blood cells, CFU, thymus index and splenocyte proliferation. DS might therefore be a potential candidate for developing new chemoprotective drugs after further in-depth experiments.

Author contributions: XL designed and guided the research; YY performed the research and wrote the manuscript; SX performed the research; QX performed the research; YG designed the research; AS analyzed data and reviewed the manuscript; NW performed statistical analysis; TW supplied critical reagents; GQ collected data.

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