Son CG *et al* / Acta Pharmacol Sin 2003 Feb; 24 (2): 120-126

Induction of hemopoiesis by Saenghyuldan, a mixture of Ginseng Radix, Paeoniae Radix Alba, and Hominis Placenta extracts

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KEY WORDS Saenghyuldan; Radix Ginseng; Radix Paeoniae Alba; Placenta Hominis; hematopoiesis

ABSTRACT

AIM: To examine the efficacy of Saenghyuldan and its components, Ginseng Radix, Paeoniae Radix Alba, and Hominis Placenta extracts (SHD, GR, PRA, and HP, respectively) on the hemopoiesis in a myelosuppression model system. **METHODS:** Susceptibility to cyclophosphamide (CP) and S180 carcinoma was determined in SHD, GR, PRA, and HP-treated mice. Analysis of peripheral blood and bone marrow cells was demonstrated by changes in cell types and histopathologic examination. The expression of cytokine mRNAs involved in hemopoiesis was examined by RT-PCR. **RESULTS:** SHD and its seperated components (GR, HP, and PRA, respectively) significantly increased the survival in CP- and S180-treated mice. The hematology data demonstrated that all the agents augmented monocyte and leucocyte counts in the peripheral blood and increased bone marrow density and the ratio of leukocyte to erythrocyte in the bone marrow. These findings were positively correlated with the up-regulation of cytokine mRNA expression such as granulocyte colony-stimulating factor (GM-CSF), erythropoietin (EPO), thrombopoietin (TPO), stem cell factor (SCF), and c-Kit. **CONCLUSION:** SHD is an effective remedy for the bone marrow failure and myelosuppression occurring during chemotherapy.

INTRODUCTION

Most of the known anticancer drugs are toxic on the normal host cells as well as the cancer cells. Rapidly proliferating cells in normal tissues such as bone marrow, lymphatic system, epithelium of the gastrointestinal tract, hair follicles, and germinal epithelium are especially sensitive to cytostatic drugs. The common side-effects of these drugs are bone-marrow suppression, nausea, vomiting, diarrhea, and alopecia[1-2].

Myelosuppression is a main life-threatening side effect among them in cancer patients. Chemotherapy causes leukopenia due to damage of bone marrow progenitors. In those afflicted, invasion and growth of opportunistic bacterial pathogens are facilitated by the reduced number or function of neutrophils which play an important role in the initial defense against infections. Besides, therapeutic dosage of the drugs should be lower than effective dosage and the following treatment would be delayed to prevent granulocytopenia and thrombocytopenia, which have frequently occurring

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Received 2002-03-06 Accepted 2002-10-08

common side-effects in chemotherapy for cancer patients[1]. Recently, EPO and various colony stimulating factors (CSFs) have been used for the treatment of anemia associated with renal diseases and neutropenia of various causes. However, their effects were reported to be limited and unexpected side-effects occasionally occurred. Also they may have little ability to accelerate neutrophil or platelet recovery after extremely intensive regimens such as high-dose etoposide and cyclophosphamide (CP)[3-8].

Considering the contemporary circumstances, the development of new drugs and therapeutics for myelosuppression is absolutely required. A number of herbal agents were known to have effects on the hemopoietic system. The mechanisms by which these agents exert their hemopoietic effects are not well understood so far. Previous findings from clinical and experimental studies demonstrated that some herbal extracts or compounds had effects on animal models and patients with myelosuppression[9-12].

Saenghyuldan (SHD) has been clinically used to the patients suffering from myelosuppression in Daejeon University Oriental Medical Hospital for the past two years based on the studies showing that SHD had significant effects on the myelosuppression through the previous laboratory findings[12]. In the present study, we investigated the action mechanism of SHD in peripheral bloods and bone marrow of CP and S180 administered mice as a myelosuppression model system.

MATERIALS AND METHODS

Preparation of Ginseng Radix (GR), Paeoniae Radix Alba (PRA), Hominis Placenta (HP), and SHD GR and PRA were purchased from Daejeon University Oriental Hospital. The roots were identified and authenticated by Prof SI RIM. Voucher specimens (#GR-2001-01, #PRA-2001-01) have been deposited at the Institute of Traditional Medicine and Bioscience in Daejeon University. GR was prepared from dried root of Korean Ginseng Radix. Briefly, Dried roots of Korean Ginseng Radix were ground to powder and extracted with 25 % ethanol for 24 h. The extract was then evaporated and lyophilized. PRA was prepared from the dried fruit of Paeoniae Radix Alba by the same way with that of GR. The lyophilized HP was obtained from Dong Duck Pharmaceutical Co (Korea; H90004). SHD was received from Daejeon Oriental Medical Hospital. SHD is a mixture of equal amounts of GR, PRA, and HP, respectively. All the agents, GR, PRA, HP, and SHD, were suspended by 200 g/L in distilled water and given orally to mice.

Experimental animals Female ICR and BALB/c mice were purchased from Dae-Han Laboratory Animal Research Center (Korea) and used at 5 to 6 weeks of ages. The mice were given food (Samyang Feed Ltd, Korea) and water *ad libitum*. The mice were maintained at 22 °C and 55 % relative humidity with 12-h light/dark cycles.

Susceptibility test and the induction of myelosuppression For susceptibility study, ICR mice were intraperitoneally (ip) injected with 500 mg/kg of cyclophosphamide (CP). At one-day post-administration of CP, the mice were ip injected with Sarcoma 180 cells (S180; 2.5×10^9/L) and orally administered 200 mg/kg of GR, PRA, HP, or SHD for 9 consecutive days. Control group was administered orally with distilled water under the same condition[9]. Survival rate was determined at the end of treating period.

The model system for myelosuppression was previously described[13]. Briefly, BALB/c mice were ip injected with CP 250 mg/kg at 5 d after oral administration with 200 mg/kg of GR, PRA, HP, and SHD, respectively. Controls were administered with distilled water followed by CP treatment. Cells in peripheral blood and bone marrow (BM) were analyzed at 4 d after CP treatment.

Analysis of the cells in peripheral blood and BM Peripheral blood was obtained by retro-orbital venous plexus sampling with heparinized capillary tube (ID; 1.1-1.2 mL, Chase Scientific Glass Inc, USA). Complete blood cell counts were determined using blood cell counter (HEMAVET, CDC Technologies Inc, USA).

BM was obtained by flushing femoral bones. For the histopathological evaluation, the BM tissue was dissected and fixed in 10 % neutral-buffered formalin. The fixed samples were embedded in paraplast and sections of 4 μm were prepared. The sections were stained with hematoxylin and eosin for histopathological examination after decalcification. Concomitantly, BM cells were smeared on slide glasses, fixed with methanol and stained with Giemsa solution. We measured the ratio of leukocytes to erythrocytes in the 1×10^3 BM cells.

Determination of cytokine mRNA expression BM cells were aseptically isolated from untreated BALB/c mice. The cells were washed twice and suspended in RPMI-1640 complete media containing with *L*-glutamine, penicillin/streptomycin, HEPES 25 mmol/L, and 5 % fetal bovine serum (Sigma, USA). The cell

suspension was transferred to 6 well plate (5×10^6 cells per well), added with the indicated concentrations of GR, PRA, HP, and SHD (1 mg/L or 10 mg/L), and incubated at 37 °C in a 5 % CO_2 incubator for 4 h.

Total RNA was extracted from the cell pellet with TRIZOL (Gibco BRL, Maryland, USA) by manufacturer's recommendation. RNA sample was reverse-transcribed at 42 °C for 1 h in a 20 μL reaction mix (for 1 μg of total RNA) containing 40 U RNase inhibitor, oligo dT_{15} 0.1 μg, 0.5 mmol/L of each dNTPs, Tris-HCl 50 mmol/L (pH8.3), KCl 75 mmol/L, $MgCl_2$ 3 mmol/L, DTT 10 mmol/L, and 200 U moloney murine leukemia virus reverse transcriptase. Samples were stored at –80 °C until subjected to PCR amplification.

To determine the expression pattern of GM-CSF, EPO, TPO, SCF, and c-Kit mRNA, 1 μL of cDNA was amplified by a thermal cycler (TaKaRa, Japan) using the specific primers. The PCR mixture was made as follows; 1.5 U of *Taq* DNA polymerase (Bioneer, Korea), 3 μL of dNTPs 10 mmol/L, 3 μL of 10×PCR buffer, 1 μL of 10 pmol sense and antisense primers, and 1 μL of cDNA in 19.7 μL of ultra distilled water. PCR amplification was carried out using a protocol of initial denaturing step at 95 °C for 10 min, then 35 cycles of amplification (at 95 °C for 1 min, at 60 °C for 40 s, and at 72 °C for 1 min), and additional extension at 72 °C for 10 min. The PCR products were separated on a 2 % agarose gel in 0.5×TBE buffer and stained with ethidium bromide. PCR primers for GM-CSF, EPO, TPO, SCF, c-Kit, and β-actin were synthesized as follows.

GM-CSF primers, sense: 5'-GGCCTTGGAAGCATGTAGAG-3'; antisense: 5'-ATACTGCCCTCCAACTGTGG-3';

EPO primers, sense: 5'-TAGAAGTTTGGCAAGGCCTG-3'; antisense: 5'-CGTGTACAGCTTCAGTTTCC-3';

TPO primers, sense: 5'-CCTCTTCTTGAGCTTGCAAC-3'; antisense: 5'-AGCCCATGAGTTCCATTCAC-3';

SCF primers, sense: 5'-TAACCCTCAACTATGTCGCC-3'; antisense: 5'-TCACTAGTGTCAGATGCCAC-3';

c-Kit primers, sense: 5'-GACGTCATGAAGACTTGCTG-3'; antisense: 5'-ACAGCAGCAAAGCCTGTTGG-3';

β-actin primers, sense: 5'-ACCGTGAAAAGATGACCCAG-3'; antisense: 5'-TCTCAGCTGTGGTGGTGAAG-3'.

Statistical analysis Results were expressed as the mean±standard deviation (SD). Statistical analysis of the data was carried out by Student's *t*-test. A difference from the respective control data at the levels of $P<0.05$ and $P<0.01$ was regarded as statistically significant.

RESULTS

SHD is a cocktailed mixture of GR, PRA, and HP extracts. At first, we examined if SHD and its components had an effect on the survival of a myelosuppression-induced mice. GR, HP, PRA as well as SHD significantly increased the survival of the ICR mice treated with CP and S180 sarcoma by 20 %-30 % compared to control group (Tab 1). To demonstrate that the increased survival is related with a recovery of the suppressed hemopoiesis, hematological analysis was performed in blood before and after SHD treatment. Treatment with SHD and its components positively influenced on the increased cell numbers of monocytes and white blood cells (WBC) of the CP and S180 treated mice. It is notable that SHD and HP showed a robust augmentation in WBC and monocyte counts (Tab 2). Unlike WBC and monocytes, red blood cell (RBC), hemoglobin, and platelet in blood remained constant in the treatment with SHD, GR, HP, and PRA. These results indicated that SHD and its components increased the cell types which was involved in primary defense mechanism.

Hemopoiesis is mainly occurring in BM. To verify that the increase in WBC and monocyte cells in blood from the SHD-treated mice was due to an activation of

Tab 1. Effect of Saenghyuldan (SHD) and its each component on the susceptibility to cyclophosphamide and S180 sarcoma-treated mice. n=8 mice. Mean±SD. $^bP<0.05$ *vs* control.

Group	Mean survival days/d	T/C[1)]/%
Control	13±3	100.0
GR	16.2±2.4[b]	127.5
HP	15±4	120.6
PRA	17±4[b]	130.4
SHD	16.0±2.2[b]	125.5

1) T/C: Mean survival time of treated mice/mean survival time of control×100 %.

Tab 2. Effect of GR, HP, PRA, and SHD on the hematological changes in cyclophosphamide and S180-induced myelosuppression. *n*=6 mice. Mean±SD. $^{b}P<0.05$ *vs* control.

Test	Group	Pre-treatment	Post-treatment
10^{-9}×WBC/L^{-1}	Control	7.4±1.2	1.2±0.4
	GR	7.7±1.6	1.6±0.6
	HP	6.8±1.3	2.5±0.9^{b}
	PRA	7.3±1.4	2.0±0.6^{b}
	SHD	7.4±1.2	2.7±1.0^{b}
10^{-11}×RBC/L^{-1}	Control	8.37±0.20	8.4±0.8
	GR	8.51±0.19	8.6±0.5
	HP	8.5±0.5	8.4±0.5
	PRA	8.5±0.5	8.5±0.4
	SHD	8.6±0.4	8.5±0.4
10^{-2}×Hemoglobin/g·L^{-1}	Control	11.5±0.4	11.7±0.8
	GR	11.3±0.5	11.2±0.8
	HP	11.6±0.4	11.6±1.2
	PRA	11.2±0.6	11.6±1.0
	SHD	11.6±0.8	11.5±1.2
10^{-9}×Platelet/L^{-1}	Control	526±131	334±54
	GR	491±66	328±39
	HP	515±128	347±40
	PRA	505±14	313±74
	SHD	528±58	328±106
10^{-9}×Monocyte/L^{-1}	Control	0.40±0.03	0.05±0.02
	GR	0.37±0.05	0.15±0.08^{b}
	HP	0.34±0.06	0.16±0.06^{b}
	PRA	0.38±0.09	0.14±0.02^{b}
	SHD	0.36±0.09	0.18±0.12^{b}

hemopoiesis in the BM, histological analysis of BM was carried out. The CP and S180 treatment resulted in low density of cellularity with large vacuole in the BM. However, SHD and HP treatment produced the higher density of BM cellularity with decreased number of vacuoles compared with contol. Meanwhile, GR and PRA treatment inhibited the formation of large vacuole though they did not affect the low density of BM cellularity induced by CP and S180 administration (Fig 1). These results imply that GR, PRA, HP, and SHD activate BM functions in myelosuppression. To further characterize the effect of SHD on BM functions, the ratio of leukocytes to erythrocytes was examined in the myelosuppression-induced mice. SHD and its component HP remarkably increased the leukocytes ratio to erythrocytes (Tab 3). This result demonstrated that

Tab 3. Ratio of leukocytes to erythrocytes in the bone marrow from the cyclophosphamide and S180-treated mice. *n*=6 mice. Mean±SD. $^{b}P<0.05$ *vs* control.

Group	Ratio of leukocytes to erythrocytes/%
Control	10±7
GR	11±10
HP	37±20^{b}
PRA	16±21
SHD	44±27^{b}

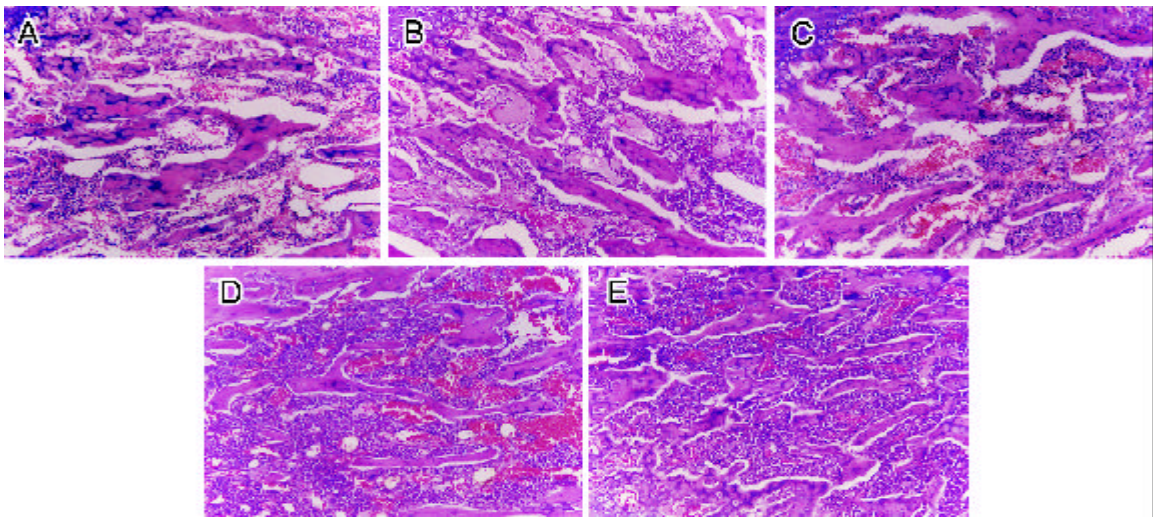

Fig 1. Histological analysis of bone marrow. The bone marrow tissues were isolated from mice treated with CP and S180 plus GR, PRA, HP, or SHD. HE stain, ×100.

the main action of SHD was a recovery of leukocytes in the BM.

To examine the SHD-induced hemopoietic cytokine production, we measured cytokine mRNA expression from BALB/c BM cells. These cells were obtained from normal BALB/c mice. GM-CSF, EPO, TPO, SCF, and c-Kit mRNA were amplified and qualitatively analyzed by RT-PCR. All the treatment groups showed an increase in GM-CSF gene expression by two times compared with control group. Among them, HP treatment showed a robust induction in GM-CSF mRNA expression by 5 times more than in control. In expression of the EPO mRNA, GR (10 mg/L) and SHD (1 mg/L) treatment enhanced a 1.5-fold induction of EPO gene expression. But most of herbal products- treated groups except for HP (1 mg/L) showed a decreased expression of TPO mRNA compared with control group. Furthermore, PRA (1 mg/L) and SHD- treated groups had no expression under the tested condition. The results were described in Fig 2. All of the herbal products-treated groups showed an enhanced expression of SCF mRNA. GR (10 mg/L), HP (10 mg/L), and SHD (10 mg/L) treatments enhanced almost 2-fold induction in the SCF gene expression. In expression of the c-Kit mRNA, GR (10 mg/L) and SHD (1, 10 mg/L) treatments enhanced 1.5-fold induction compared with control group.

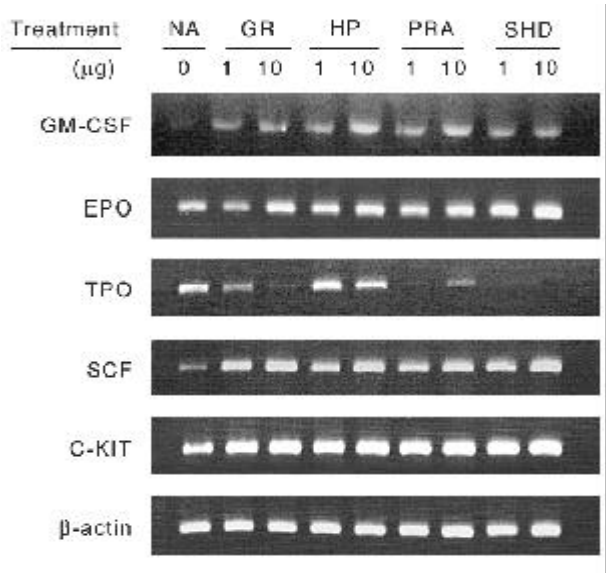

Fig 2. Expression of GM-CSF, EPO, TPO, SCF, and c-Kit mRNA in the bone marrow cells treated with GR, HP, PRA, and SHD. Bone marrow cells were isolated and cultured in the presence of 1 or 10 mg/L of the indicated agents. Total RNA was purified and subjected to RT-PCR analysis as described in Materials and Methods. One of the three independent experiments was shown.

DISCUSSION

Myelosuppression has been known as one of the major drawbacks in cancer chemotherapy. Since toxicity associated with neutropenia and thrombocytopenia often limits the dose of chemotherapy, isolation of agents that could accelerate granulocyte and platelet recovery may prove to be clinically beneficial[1,9].

The present study shows that SHD has positive functions on up-regulating monocytes and white blood cells that involved in host defense mechanisms. According to the experiments using SHD and its component extracts, this function of SHD might be due to most likely HP in spite of requiring further characterization. There are few reports on HP studies though it has been widely used as an oriental medicine for various diseases. Our results that SHD and HP stimulate hemopoiesis in myelosuppression model provide the first scientific clues on the usage of HP.

A stem cell is defined as a cell with the ability both to self renew and to differentiate. Stem cells can sustain self-replication for approximately 50 cell divisions, which are sufficient to maintain the hemopoietic system of an adult for the whole life span. The self-replication and proliferation of BM can be maintained in combination of soluble cytokines or growth factors. Primitive hemopoietic stem cells do not proliferate in response to any single cytokine, but their growth is promoted by combinations[14-18]. Some cytokines have little or no independent effect on hemopoiesis but act synergistically with others. SCF is one of the most potent CSF acting synergistically. It interacts with many other cytokines to promote growth of myeloerythroid and lymphoid stem cells and hence increases the production of all blood cells. SCF activates multiple signaling pathways and these pathways lead to a variety of biological responses. c-Kit is a receptor tyrosine kinase that binds SCF, closely related to the receptors for platelet-derived growth factor and colony-stimulating factor[14, 20-21]. GM-CSF has an ability to stimulate proliferation and maturation of granulocytes and macrophage myeloid cells. This cytokine also stimulates various functional activities of leukocytes including cytotoxicity of granulocytes and macrophages, phagocytic activity and degranulation of neutrophils. But in the previous studies, GM-CSF treatment resulted in the decrease of platelet. Also GM-CSF had little effect on

hemopoietic recovery after high-dose etoposide and cyclophosphamide treatment[5,22].

Several cytokines have been shown to increase platelet count in myelosuppressed animals. For example IL-2 and IL-6 had an positive effect on the therapy against thrombocytopenia. However, the effect was slowly occurring and IL-2 was required for a longer time. In the recent reports, TPO had favorable effects on thromcytopenia without any apparent hematologic or other toxicities[23].

Hemopoiesis is controlled by at least 30 known cytokines. There is no doubt that these cytokines related to hemopoiesis are clinically beneficial. Cytokines regulate each other in their production and activity through competition, synergism, and mutual induction, resulting in a complex network of cytokine cascades and regulatory circuits with positive and negative feedback. In addition, other types of biologic mediators have agonistic or antagonistic effects on cytokine activities. Owing to the complexity of cytokines interaction, the therapeutic use of these agents is still in its infancy[17]. Hence it might be better to release cytokine not through artifical infusion but through natural production in human body.

We previously demonstrated that administration of SHD into normal mice showed a protective effect on the BM failure induced by CP and irradiation. This study also showed that oral administration of SHD significantly inhibited a reduction of leukocytes after CP-treatment and accelerated recovery from CP-induced leukopenia[12].

SHD contains 3 species of medicinal herbs such as GR, PRA, and HP. Among the herbs, HP-treated group showed an obvious effect on all of the hemopoietic lineages tested herein except erythroid. Especially, GM-CSF mRNA expression was enhanced in the HP-treated group compared with control. Even though direct infusion of GM-CSF has significantly reduced neutropenia, the GM-CSF did not affect platelet recovery and had no significantly positive impact on thrombocytopenia. On the contrary to the direct infusion of GM-CSF, HP treatment increased the expression of TPO and platelet count as well as GM-CSF expression as demonstrated by RT-PCR. However basal level of WBC count showed little difference compared with control group. GR treatment enhanced more than 1.5-fold in gene expression of EPO compared with other treated groups. These results implies that GR has a significant effect on hemopoiesis in erythroid lineage. But the GR treatment had no significant effect on neutropenia. PRA-treated group did not show a significant effect on the BM and peripheral blood counts. However, PRA treated group showed a significant effect on the survival of the S180 injected mice compared with control and other treated groups.

Taken together, we demonstrated that SHD and their components functioned in an enhancement of neutrophils and monocytes in the BM as well as peripheral blood in the chemically damaged mice. The effect of SHD might resulted from the regulation of the cytokine expression which is responsible for hemopoiesis such as GM-CSF, EPO, SCF, and c-Kit. These results suggest that SHD and their components are good candidates for new drugs or therapeutics on chemotherapy-associated myelosuppression.

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