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Original Article

Effects of *Saenghyul-dan* (SHD) on the Myelosuppression Induced by 5-Fluorouracil

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Objective : This study aimed to investigate the effects of SHD on myelosuppression using an animal model for therapeutic evidence supporting clinical positive results.

Methods : After determining the optimal concentration of 5-FU as 300 ㎎/㎏ for developing the mouse model, ICR mice or BALB/c mice were administered with SHD, and several blood parameters, including hematopoietic cytokines, colony forming activity and histological findings, were examined to evaluate the effects.

Results : SHD restored the WBC and hemoglobin, and showed effects on maintaining body weight, producing GM-CSF and IL-3 and stem cell colony forming activity in accordance with histological relative entirety on bone marrow.

Conclusion : SHD is an herbal drug having therapeutic effects on myelosuppression. Thus, it could be prescribed to cancer patients undergoing chemo-therapies or radio-therapies in the process of cancer treatment.

Key Words : *Saenghyul-dan* (shēngxiědān), myelosuppression

Introduction

Bone marrow dysfunction is one of the most frequent and critical adverse effects to conventional cancer therapeutics such as chemotherapy or radiotherapy. Moreover, the decreased immunity from these conditions is known as a crucial complication often requiring ending the treatment early or reducing the curative dose and sometimes even leading to death. It is also linked to cancer metastasis or recurrence. Therefore, novel drugs have been intensively sought which selectively destroy only the cancer cells using a molecular targeting strategy based on genomic specificity among tumors or normal cells. However, most existing treatments are still being applied to patients, with the accompanying unwanted side effects and lowered efficacy[1-8].

On the other hand, integrative treatment has been adapted as an alternative way to accomplish the final goal of cancer patients, including enhancement of life through improvement of life quality and prolonged life. Accordingly, complementary medicine including oriental medicine is becoming popular in the fields of cancer management in both Eastern and Western countries. In addition, oriental herbal medicine has been a potential candidate for myeloprotective drugs

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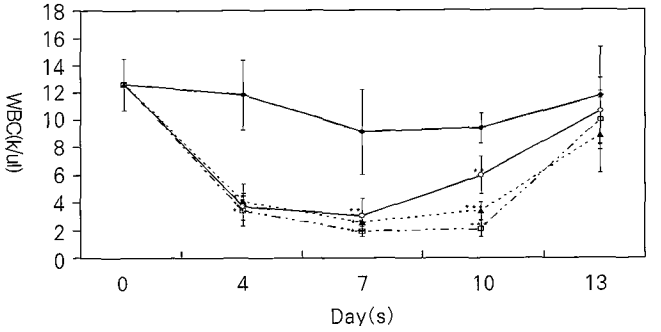

Fig. 1. WBC count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (200, 250, 300 mg/kg). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 5 mice. Naive, ◆ ; 5-FU 200 mg/kg, ○; 5-FU 250 mg/kg; 5-FU 300mg/kg, □. Statistical significance was compared with naive group by *t*-test. (**: p<0.01, ***: p<0.001).

against conventional therapeutics-induced myelosuppression[1,2,9,15].

Saenghyul-dan (SHD) is a herbal prescription that has been used for patients suffering from blood deficiency and lower vital energy of kidney, spleen and liver. According to oriental medical theory, these three organs are responsible for balancing the essential components of blood and Qi (氣). This drug has also been studied for protective or therapeutic effects on bone marrow functional suppression in an animal model. This drug has been prescribed for cancer patients having leukopenia or anemic symptoms and has shown worthwhile effects at Daejeon Oriental Hospital since 2001[16].

In this study, SHD was investigated in order to gather animal-based evidence to support positive clinical results from cancer patients.

Materials and Methods

1. Materials

Ginseng Radix and *Paeoniae Radix Alba* were

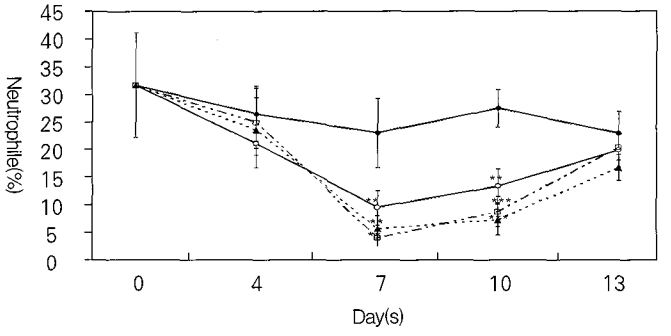

Fig. 2. Neutrophile count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (200, 250, 300 mg/kg). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 5 mice. Naive, ◆ ; 5-FU 200 mg/kg, ○; 5-FU 250 mg/kg; 5-FU 300mg/kg, □. Statistical significance was compared with naive group by *t*-test. (**: p<0.01, ***: p<0.001)

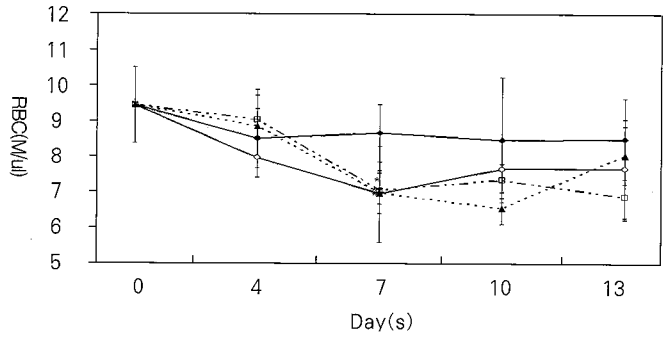

Fig. 3. Red blood cell count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (200, 250, 300 ㎎/㎏). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 5 mice. Naive, ◆ ; 5-FU 200 mg/kg, ○; 5-FU 250 mg/kg; 5-FU 300mg/kg, □. Statistical significance was compared with naive group by *t*-test. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$)

acquired from Daejeon University Dunsan Oriental Hospital. Briefly, 100g of *Ginseng Radix* was washed with DW and boiled in 1L DW for 2 hr. The *Ginseng Radix* extract (GRE) was centrifuged to remove debris, filtered, evaporated and lyophilized. *Paeoniae Radix Alba* extracts (PRAE) were prepared in the same way as the *Ginseng Radix* extract. The lyophilized Hominis placenta was obtained from Dong Duck Pharmaceutical Co. (Korea; H90004). It was hydrated in pepsin solution (5g/L) at pH 2.0 for 2 h. The hydrated solution was adjusted to pH 7.0 with NaOH and centrifuged for 15 min at 1500 rpm. Supernatants were lyophilized to make Hominis placenta extract (HPH). SHD was made by mixing GRE, PRAE and HPH in the ratio of 1:1:2. 5-FU was obtained from Choongwae Pharmaceutical Co. (Korea).

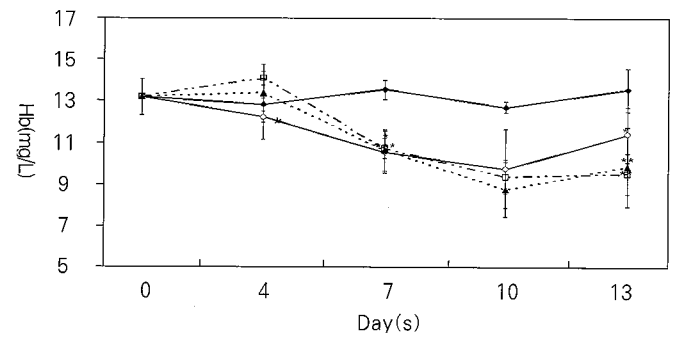

Fig. 4. Hemoglobin count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (200, 250, 300 ㎎/㎏). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 5 mice. Naive, ◆ ; 5-FU 200 mg/kg, ○; 5-FU 250 mg/kg; 5-FU 300mg/kg, □. Statistical significance was compared with naive group by *t*-test. (*: not significant, **: $p<0.05$, ***: $p<0.001$)

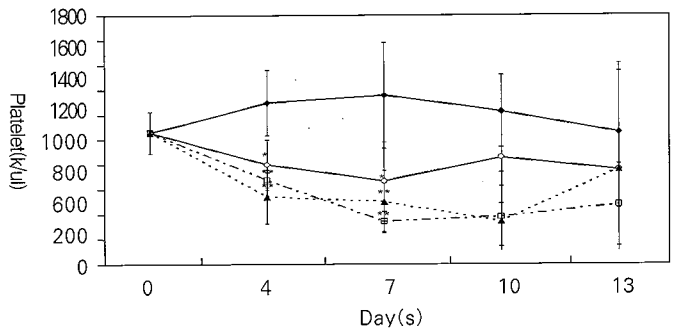

Fig. 5. Platelet count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (200, 250, 300 ㎎/㎏). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 5 mice. Naive, ◆ ; 5-FU 200 mg/kg, ○; 5-FU 250 mg/kg; 5-FU 300mg/kg, □. Statistical significance was compared with naive group by *t*-test. (*: p<0.05, **: p<0.01, ***: p<0.001)

2. Experimental animals

Male ICR and BALB/c mice were purchased from Koatech Co. (Pyongtaek, Korea) and used at 7 to 8 weeks of age. The mice were given food (Samtako, Korea) and water *ad libitum*. The mice were maintained at 22℃ and 55% relative humidity with 12 h light/dark cycles. After acclimatization to their new environment for at least 1 week, mice were used for each experiment.

3. Myelosuppression induction

In the initial phase of the study, six ICR mice from each group were intraperitoneally injected with 200, 250, 300 ㎎/㎏ of 5-FU or normal saline for control to decide the optimal concentration of 5-FU for the myelosuppression model. Hematologic parameters were monitored on the 0, 4th, 7th, 10th and 13th days after 5-FU injection.

To study SHD, six ICR mice from each group

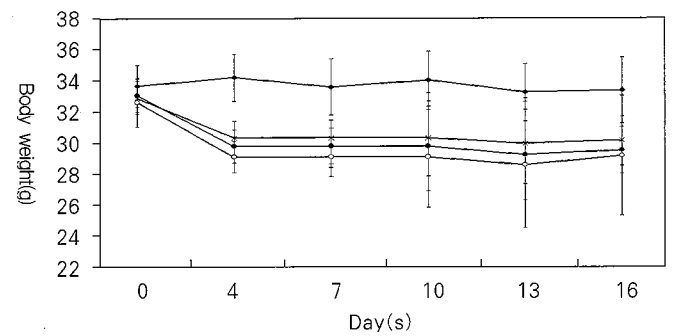

Fig. 6. Body weight changes in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (300 ㎎/㎏). Two days after administered with SHD (50 ㎎/㎏, 200 ㎎/㎏). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Naive, ◆ ; Induced, ○; SHD 50mg/kg, ×; SHD 200mg/kg, ●. Statistical significance was compared with induced group by *t*-test. (*: not significant, **: p<0.05, ***: p<0.001)

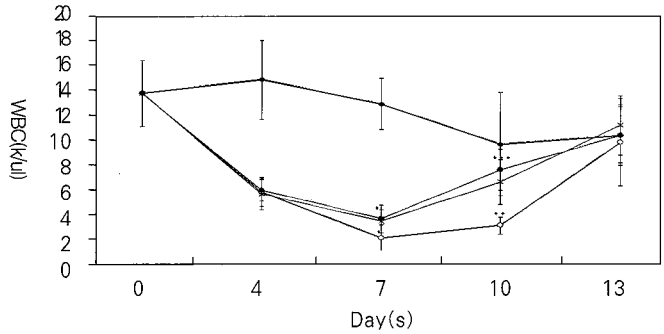

Fig. 7. WBC count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (300 ㎎/㎏). Two days after administered with SHD (50 ㎎/㎏, 200 ㎎/㎏). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Naive, ◆ ; Induced, ○; SHD 50 mg/kg, ×; SHD 200 mg/kg, ●. Statistical significance was compared with induced group by *t*-test. (*: p<0.05, **:p<0.01, ***: p<0.001)

except the normal group were intraperitoneally injected with 300 ㎎/㎏ of 5-FU. Two days later, the mice were orally administered SHD (50 ㎎/㎏, 200 ㎎/㎏), or water for control, once a day for 11 consecutive days.

4. Cell counts in the peripheral blood

Blood was obtained by retro-orbital venous plexus sampling with heparinized capillary tube (I.D.; 1.1~1.2㎖, Chase Scientific Glass Inc., U.S.A.). Complete blood counts were determined using blood cell counter (HEMAVET, CDC Technologies Inc., U.S.A.) on the experiment days 0, 4, 7, 10 and 13 after 5-FU injection.

5. Immunoassay for hematopoiesis-related cytokines

To know the effects of SHD on induction of several cytokines, another five ICR mice from each group except the normal group were int-

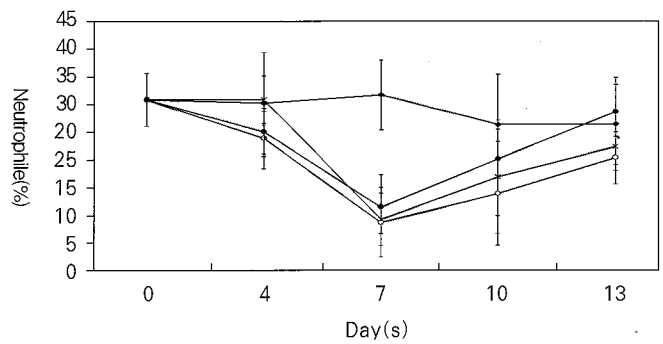

Fig. 8. Effect of SHD on the Neutrophil count in the 5-FU treated Mice ICR mice were intraperitoneally injected with 5-FU (300 ㎎/㎏). Two days after administered with SHD (50 ㎎/㎏, 200 ㎎/㎏). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Naive, ◆ ; Induced, ○; SHD 50 mg/kg, ×; SHD 200 mg/kg, ●. Statistical significance was compared with induced group by *t*-test. (*: p<0.05)

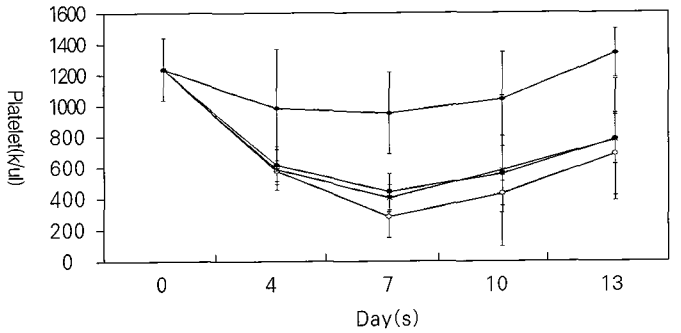

Fig. 9. Effect of SHD on the Platelet count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (300 mg/kg). Two days after administered with SHD (50 mg/kg, 200 mg/kg). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Naive, ◆ ; Induced, ○; SHD 50 mg/kg, ×; SHD 200 mg/kg, ●. Statistical significance was compared with induced group by *t*-test.

raperitoneally injected with 300 mg/kg of 5-FU. Two days later, the mice were orally administered SHD (50 mg/kg, 200 mg/kg), or water for control, once per day for 5 days. After collection of total serum from each at the 7th day of the experiment, interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and thrombopoietin (TPO) levels were measured using Quantikine M, ELISA Kit (R&D system, Inc., U.S.A.)

6. Colony forming assay from haemopoietic stem cells

Three male BALB/c mice from each group (control, SHD 50mg/kg and 200mg/kg) were intraperitoneally injected with 5-FU (125mg/kg), or with normal saline for the control. From two days later, they were orally fed SHD, or water for control, for 5 days. On day 7 of the experiment, the one side femur from each mouse was removed to collect bone marrow cells by cold D-PBS.

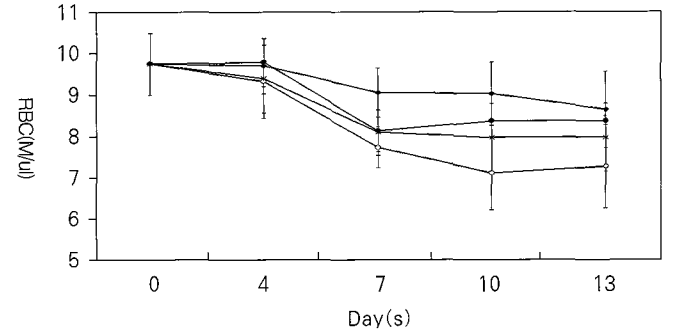

Fig. 10. Effect of SHD on the Red blood cells count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (300 mg/kg). Two days after administered with SHD (50 mg/kg, 200 mg/kg). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Naive, ◆ ; Induced, ○; SHD 50 mg/kg, ×; SHD 200 mg/kg, ●. Statistical significance was compared with induced group by *t*-test. (*: $p<0.05$)

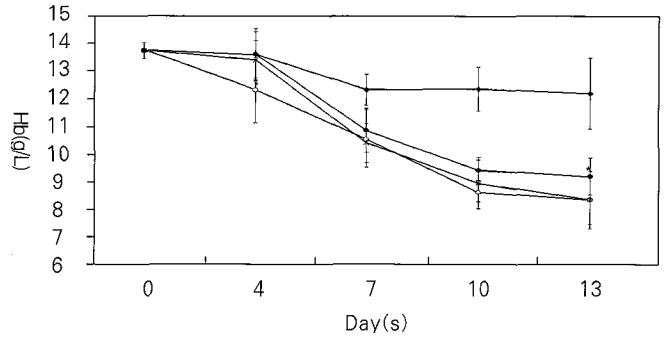

Fig. 11. Effect of SHD on the Hemoglobin count in the 5-FU treated Mice. ICR mice were intraperitoneally injected with 5-FU (300 mg/kg). Two days after administered with SHD (50 mg/kg, 200 mg/kg). Hematologic parameters were monitored on 0st, 4th, 7th, 10th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Naive, ◆ ; Induced, ○; SHD 50 mg/kg, ×; SHD 200 mg/kg, ●. Statistical significance was compared with induced group by *t*-test. (*: $p<0.05$)

These cells were used for isolation of haemopoietic stem cells using a gradient method (Histopaque-1077, Sigma Inc.). Isolated stem cells were mixed (2000 cells/ml) into specialized media (Methocult GF M3434, StemCell Tech.) and cultured in 35 mm grid dish as triplet (1.2 ml/ dish) per group at 37 ℃, 5% CO2, humidified incubator for 7 days. On the final day, the colony number of each dish was counted under inverted microscope and classified into CFU-GM (colony forming unit granulo/monocyte) or CFU-E (colony forming unit erythrocyte).

7. Histological analysis of BM

Histological analysis of BM was performed on the 7th day after 5-FU (125 mg/kg) treatment in male BALB/c mice using another side femur after bone marrow cell collection for colony forming assay. For the histomorphological evaluation, the BM tissue was dissected and fixed in 10% neutral-buffered formalin. After decalcification, fixed samples were embedded in paraplast and sections

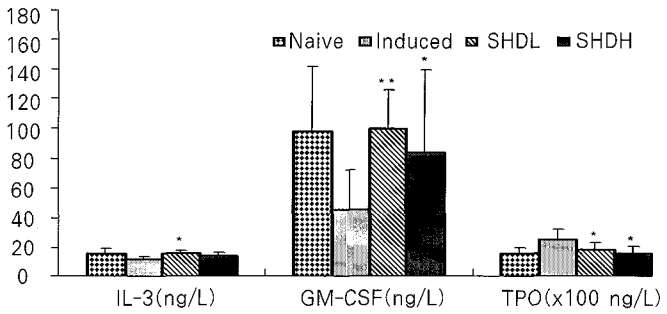

Fig. 12. Immunoassay for hemopoiesis related cytokines. ICR mice were intraperitoneally injected with 5-FU (300 mg/kg). The mice were administered with SHD (50 mg/kg, 200 mg/kg) or water for control just 2 day after 5-FU treatment. Immunoassay of cytokines was measured on 7th day after 5-FU treatment. n=5. Mean±SD. Statistical significance was compared with induced group by *t*-test. (*: $p<0.05$, ** $P < 0.001$)

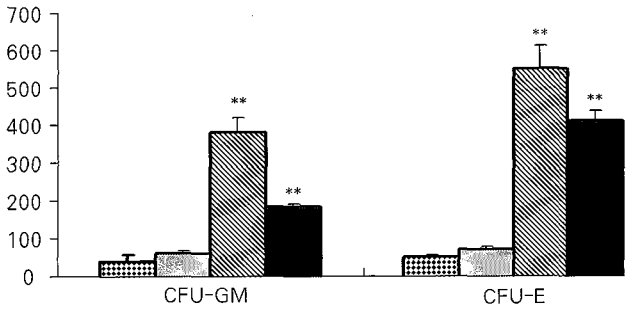

Fig. 13. Colony forming units of bone marrow cells in myelosupressed mice. BALB/c mice were intraperitoneally injected with 5-FU (125 mg/kg). The BALB/c mice were administered with SHD (50, 200 mg/kg). Bone marrow cells were isolated 7 days after 5-FU injection. Statistical significance was compared with induced group by *t*-test. (*: $p<0.05$, **: $p<0.01$)

of 4 ㎛ were prepared. The sections were stained with hematoxylin and eosin for histopa- thological examination.

8. Statistical analysis

Results were expressed as the mean ± standard deviation (S.D). 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.

Result

1. Modeling 5-FU-induced myelosuppression

In order to choose the optimal concentration of 5-FU in construction of a mouse-based animal model for chemotherapy-induced myelosuppression, ICR mice were intraperitoneally injected with three different concentrations (200, 250 or 300 mg/kg). Thereafter, hematologic parameters were

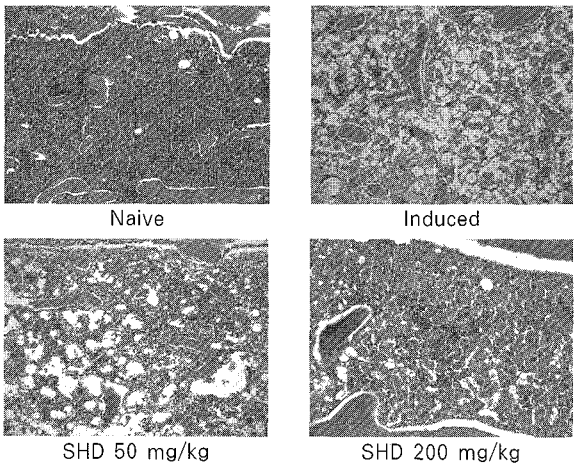

Fig. 14. Histological analysis of bone marrow. BALB/c mice were intraperitoneally injected with 5-FU (125 mg/kg). The mice were administered with SHD (50, 200 mg/kg). The bone marrow tissues were isolated 7 days after 5-FU injection. H&E stain. X 200.

monitored on the 0, 4th, 7th, 10th and 13th day after 5-FU injection.

As shown in figures 1 to 5, mice had significantly decreased bone marrow cell population, such as WBC, RBC and platelets, and other parameters like neutrophils and hemoglobin. Moreover, they showed the myelosuppression pattern in a dose-dependent manner.

In this model, no mouse death was observed throughout the experiment. Accordingly, 300 mg/kg of 5-FU was chosen for following experiments in study of SHD.

2. Effect of SHD on body weight in myelo-suppressed mice

Body weight of mice in each group was continuously monitored every three days after first treatment with 5-FU. As shown in figure 6, a single injection with 300 mg/kg of 5-FU rapidly caused the reduction of body weight for all groups comparing to the control group. During all experiment days, mice gave loose feces and had unpolished color of body hair.

In the analysis of body weight, SHD generally showed a mild effect on protection against weight loss comparing to the control group throughout the 16 days

3. Effect of SHD on hemato-parameters in myelosuppressed mice

After single treatment with 300 mg/kg of 5-FU, hematologic parameters were monitored every 3 days. As shown in figures 7, 8, and 9, the number of WBC and platelets became lowest at day 7, and gradually recovered by the final day, especially for WBC and neutrophils.

Figure 7 demonstrates the significant therapeutic effect of SHD on mouse myelosuppression induced by 5-FU at day 7 and 10 measurements. As expected, neutrophils consisting of 40-60% of total WBC showed a very similar pattern with WBC. Figure 8 shows the decreased number of platelets in the three groups treated with 5-FU. And, although SHD seemed likely to have an effect on recovering this, there was no statistical difference between the SHD and control groups.

On the other hand, RBC and hemoglobin decreased continuously until the final day, as in figures 10 and 11. This might be due to repeated sampling of the blood through retro-orbital venous plexus. As shown in figure 10, lower concentrations of SHD (mg/g) showed a significant higher value than control only at day 13. After a single treatment with 300 mg/kg of 5-FU, hematologic parameters were monitored every 3 days. As shown in figures 7, 8, and 9, the number of WBC and platelets became lowest at day 7, and gradually recovered by the final day, especially for WBC and neutrophils.

4. Effect of SHD on hematopoietic cytokines

In order to know if SHD may work through induction of hematopoietic cytokines, another experiment was performed for 7 days. After injection with 5-FU (300 mg/kg), five mice were fed SHD from the 3rd day of the experiment for 5 days, and serum levels of IL-3, GM-CSF and TPO were measured using ELISA Kit.

As shown in figure 12, 5-FU treatment suppressed IL-3 and GM-CSF production, and SHD restored it. Contrarily, serum TPO level was higher in control than normal mice or SHD administered groups.

5. Effect of SHD on colony formation of hematopoietic stem cell

To further confirm effects of SHD on bone marrow deficiency, three BALB/c mice were

administered with SHD (50, 200 mg/kg) for 5 days after treatment with 5-FU (125 mg/kg). Isolated hematopoietic stem cells were cultured for 7 days to compare the capability of colony formation.

The top picture of figure 13 presents two main classifications of bone marrow derived colony formation. CFU-GM looks like a more scattered shape while CFU-E presents a compact round yellow-reddish shape under inverted microscope.

As shown in the bottom picture of figure 13, SHD administration significantly increased the number of colonies compared to the control and normal groups. The control group also showed a slightly higher number of colonies than the normal group.

6. Histological examination

Histological analysis of BM was performed on the 7th day after 5-FU (125 mg/kg) treatment in male BALB/c mice using another side femur after bone marrow cell collection for colony forming assay.

As shown in figure 14, three groups treated with 5-FU had decreased cell population in bone marrow while these groups contain many lipid follicles. However, SHD groups restored to some degree in 50 mg/kg and very significantly in 200 mg/kg.

Discussion

It is well known that conventional cancer therapeutics are toxic on normal host cells as well as cancer cells. Most of all, rapidly proliferating cells in bone marrow are especially sensitive to cytostatic chemotherapy or radiotherapy. Accordingly, bone-marrow suppression is one of the most common side-effects; this myelosuppression has sometimes been accepted as an inevitable and life-threatening adverse effect in the process of cancer treatment[1,2,18].

Moreover, reduced number or function of immunocytes or platelets and lowered level of blood hemoglobin are responsible for a high incidence of opportunistic bacterial infections, nonspecific bleeding and deficiency of energy. These therapeutics-derived poor conditions can be major disruptions to effective treatment with optimal therapeutic dosage or for sufficient therapeutic period. Of course, they are also main factors lowering quality of life for cancer patients[1-3].

Many alternative strategies have been tried, such as tumor-specific molecular targeting drug development, lower-dosage and highly focused irradiation, and management with GM-CFU or various colony stimulating factors (CSFs). However, their lower efficiency and clinical limitations still require new drugs and therapeutics for myelosuppression. Traditionally, a number of herbal drugs were known to have effects on the hemopoietic system, and several prescriptions have been reported to have therapeutic or protective properties for leukopenia or anemic conditions.[17,18,20,21]

Saenghyul-dan (SHD) has been used clinically for patients suffering from myelosuppression in Daejeon University Oriental Medical Hospital since 2001. Previous studies partially showed that SHD had significant effects on myelosuppression through laboratory findings[19]. The present study aimed to revalidate the therapeutic effects of SHD using another modified animal model.

First, we sought to determine which concentration of 5-FU could be optimal for the myelosuppression model using male ICR mice. The single intraperitoneal injection with 200, 250 and 300 mg/kg of 5-FU showed a reduced population of bone marrow

cells (Fig. 1-5). There were dose-dependent effects and no mice died during the 13-day experiment, so we decided to use the highest concentration for the following animal model.

Next, mice were fed SHD for 11 days from the third day after 5-FU treatment to see the effects on peripheral blood. In the periodic measurement of blood parameters, they showed the lowest value at experimental day 7, followed by gradually recovering WBC and platelets (Fig. 7-9). In this model, SHD showed a therapeutic effect significantly only at days 7 and 10 because all groups were restored by day 13. Also, mice treated with 5-FU were observed with decreased body weight compared to the non-treated normal group in monitoring until 3 days after final SHD administration. Although there were no statistically significant differences between the groups, the SHD group maintained a moderately increased weight over the control group (Fig. 6). RBC numbers were generally coincident with hemoglobin value. They became lower according to time of removal of blood to measure the blood parameters. The short and long half-life time may explain the difference among WBC, platelet and RBC.

Another reason could be the pharmaceutical properties of SHD working on hematopoietic stem cells. To address this question, three hematopoietic cytokines were measured. Mostly, GM-CSF was inducted more significantly than IL-3 or TPO (Fig. 12). This result might be accordance with WBC recovering effect (Fig. 7).

However, the other experiment, colony forming assay, was harmonious with this because the activated number of CFU-GM and CFU-E showed a very similar pattern (Fig. 13). Results could be morphologically confirmed by last observation in bone marrow section. 5-FU severely destroyed the bone marrow cells and SHD moderately repaired it (Fig 14).

In summary, these results clarify that SHD has a therapeutic effect on myelosuppression induced by 5-FU in mice model, and this effect may be exerted through activation of hematopoietic cytokine production. In the future, further detail of the mechanism and compositional analysis of SHD should be studied through controlled clinical evaluation.

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