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Effects of Ginseng Components on Immunotoxicity of Cyclophosphamide

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Abstract□Ginseng components were examined for their ability to modify immunotoxicity of cyclophosphamide. Ginseng polysaccharide fraction (FO, 300 mg/kg) inhibited decreases in the ratio of spleen to body weight, white blood cells (WBC) count and the number of plaque forming cells (PFC) induced by cyclophosphamide (50 mg/kg, i.p.), and increased these variables in normal mice. Ginseng saponin fraction (50 mg/kg) showed to increase hemoglobin content as well as the number of PFC/spleen in normal mice. On the other hand, panaxytriol (20 mg/kg) prevented decrease in WBC count by cyclophosphamide. Neither saponin fraction nor panaxytriol had any significant effect on the number of PFC and antibody titers in cyclophosphamide-treated mice. These results suggest that ginseng polysaccharide fraction may reduce the immunotoxicity of cyclophosphamide and may be effective in stimulating immune function in normal mice.

Keywords□*Panax ginseng*, Saponin, Polysaccharides, Panaxytriol, Cyclophosphamide, Immunosuppression.

Introduction

Anticancer drugs are cytotoxic to cancer cells as well as normal cells.[1] Thus, one of their major side effects is severe immunosuppression and the damage during extended treatment limits their clinical usefulness. Cyclophosphamide (CY) converted to alkylating metabolite by hepatic cytochrome P-450 is frequently used to treat lymphosarcoma and Hodgkin's disease, as well as breast, ovarian and lung caner and used also as potent immunosuppresive agent.[2-6] It was reported that CY depressed humoral immune responses severely, whereas it enhances the cellular immune response as measured by delayed hypersensitivity.[7-13] The immunoenhancing effects of CY have been ascribed to a selective inhibition of Ts generation. It has been reproted that extract or powder of *Panax ginseng* reduced the side effects of anticancer drugs, such as mitomycin C, 5-fluorouracil, cyclophosphamide and vinblastin.[14-16]

In this study, modulatory effects of ginseng saponin fraction, polysaccharide fraction and panaxytriol on white blood cells count, hemoglobin content and humoral immune responses, such as plague forming cells and antibody titers were examined in ICR mice treated with or without CY.

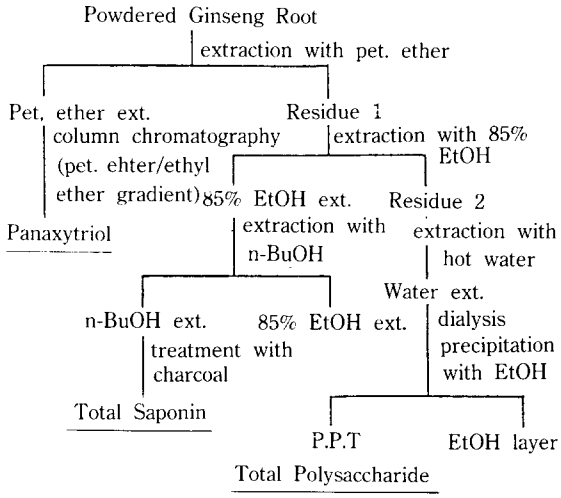
Materials and Methods

1. Animals

Male ICR mice 5-6 weeks old, weighing 24-28g, were supplied from our Animal Breeding Unit. They were housed in a room at 22± 2℃ with 55± 5 % relative humidity and given a defined laboratory rodent chow (Sam Yang Inc. Ltd.) and tap water *ad libitum*.

2. Ginseng Sample Preparation

As shown in Scheme 1, saponin fraction, panaxytriol and polysaccharide fraction (FO) were prepared from powdered Korean red ginseng (obtained from Korea Tobacco and Ginseng Corporation). Panaxytriol was isolated by the method of Ann and Kim.[17] Purified saponin fraction which was filtered with MeOH on activated charcoal column was prepared by the method of Sanada *et al.*.[18] Water-soluble polysaccharide fraction (FO) was prepared as described previously.[19]



Scheme 1. Ginseng Sample Preparation

3. Experimental Schedule

Ginseng samples, saponin fraction and FO dissolved in sterile physiological saline and panaxytriol dissolved in 50% (v/v) PEG 400/physiological saline, were intraperitoneally administered to mice from day 1 to day 6. Cyclophosphamide (Sigma Chemical Co.) was dissolved in sterile physiological saline immediately before use and intraperitoneally injected 3hr after ginseng sample administration as a single dose at 50 mg/kg on day 3. At the next day, mice were immunized with 1×10^8 sheep red blood cells (SRBC, purchased from Korea Media, Korea) injected via tail vein. On day 7, the number of plaque forming cells and antibody titers were determined to assess effects of ginseng ccomponents on immune funtion. In addition, white blood cells count and hemoglobin content were measured.

4. Preparation of Spleen Cell Suspension

The spleen was aseptically removed from each mouse after anesthesia with ether vapor and gently teased between the frosted ends of two microscope slides and passed through a nylon mesh in ice-cold Hank's Buffered Salt Solution (HBSS, pH 7.2). After centrifugation at 400g for 5 min, cell pellet was suspended in 5 m*l* of Tris-buffered 0.83% NH_4Cl (pH 7.2) and incubated at 37℃ for 5 min. The single cells obtained were washed twice with ice-cold HBSS by centrifugation at 400g for 5 min, resuspended and counted using a hemacytometer.

5. Hemolytic Plaque Forming Cell (PFC) Assay

At the 7th day of ginseng sample administration, the PFC response to SRBC was determined by the method of Chunningham and Szenberg[20] with a slight modification. Two microchambers with the volume capacity of 45 μ*l* covered with coverglass were made on the slide glass. The suspension of indicator red cells in HBSS was prepared with 500 μ*l* of 50% (v/v) SRBC and 1000 μ*l* of guinea pig complement (Sigma Chemical Co.) and stood in an ice bath for 30 min. Reaction mixture was composed of 435 μ*l* of spleen cell suspension and 100 μ*l* of suspension of indicator red cells. The mixture was packed into microchambers and then microchambers were sealed with paraffin/wax (1:1). After incubation at 37℃ for 1 hr, the number of hemolytic plaques was determined under a low power microscope. PFC/10^6 spleen cells and PFC/spleen were calculated by the following equations:

$$\text{PFC}/10^6 \text{ spleen cells} = \frac{N}{C\,V_m\,a} \times 10^6$$

$$\text{PFC/spleen} = \frac{\text{PFC}}{10^6 \text{ spleen cells}} \times C\,V_s$$

a: the ratio of spleen cell suspension in reaction mixture (435/535)

N: the number of plaque observed in a microchamber

C: the count of spleen cells in 1 m*l* of spleen cell suspension

V_m: the volum of incubation mixture filled into a microchamber

V_s: total volume of spleen cell suspension (m*l*)

6. Antibody Titration

Blood collected from the posterior vena cava after anesthesia with ether vapor was centrifuged in order to obtain serum at 3000 rpm for 10 min. The serum was inactivated at 56℃ for 30 min. After twofold serial dilution of serum with HBSS (pH 7.2) in 96-well microplate (Green Cross Medical Corp. Korea), 0.025 m*l* of 5% (v/v) SRBC in HBSS (pH 7.2) was added to each diluted serum and mixed. Hemagglutinin(HA) titer was assayed after incubating at 37℃ for 18 h. Hemolysin(HY) titer was assa-

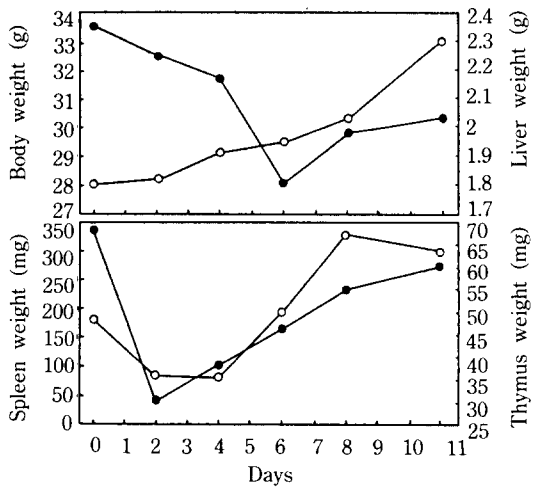


Fig. 1. Effects of cyclophosphamide on body (○), liver (●), spleen ((○) and thymus weights (●) in ICR mice. Cyclophosphamide was intraperitoneally injected as a single dose (100 mg/kg) on day 0.

yed by adding 0.025 m*l* of diluted (1:20) guinea pig complement to the mixture of serial diluted serum and SRBC, and incubating at 37℃ for 1 hr.

7. Determination of White Blood Cells (WBC) count and hemoglobin (Hb) content

Blood collected from the posterior vena cava was displaced into EDTA-2Na tube (Green Cross Medical Corp. Korea). WBC count and Hb content were determined using Coulter Counter (Coulter Electronics LTD., UK).

8. Statistical Analysis

Student's t-test was used for statistical analysis.

Results

1. Determination of Optimal Experimental Schedule

Because it has been reported that effects of CY on immune responses depend on time after and dose of the drug administration,[21] to determine optimal experimental model, effects of CY with time and dosages on body weights, immunoorgan weights, WBC counts and Hb contents as well as immune reponses were examined. When CY was intraperitoneally injected to mice at a dose of 100 mg/kg on day 0, liver, spleen and thymus weights showed a marked drop on day 6, 2-4 and 2, respectively and thereafter liver and thymus weights progressively recovered (Fig. 1). But spleens were greatly enlarged on day 8, almost doubling the normal weight and on day 11, returned gradually to normal. As shown in Fig. 2, the decrease in WBC count and Hb content was maximum on day 4 and WBC count was higher than that of the normal on day 8 as changes in spleen weights. On day 11, WBC counts and Hb contents showed to be normal. The changing pattern of marked decrease, recovery, overshoot above the normal level, and gradual normalization of spleen weight with time was consistent with the results of Chung *et al.*[22] However, the changing pattern of WBC count differed from the observation by Ha and Chung[9] that WBC count recovered progressively to normal without overshoot. To investigate when the number of direct PFC to SRBC which represents the number of IgM-producing lymphocytes and antibody titers show to be maximum, these variables were determined at indicated day in Table 1 after immunization with SRBC. The maximum response in PFC and antibody titers were elicited on day 4 and 6, respectively.

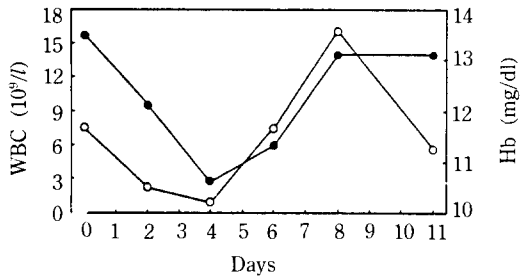


Fig. 2. Effects of cyclophosphamide on white blood cells (○) and hemoglobin ((●) in ICR mice. Cyclophosphamide was intraperitoneally injected as a single dose (100 mg/kg) on day 0.

From these results, the maximum decreases in spleen weight, WBC count and Hb content caused by CY were observed on day 4 after CY injection. Also the number of PFC was maximumly increased after 4 days of immunization.

Therefore, effects of CY on the number of PFC and antibody titers were determined by following experimental schedule; Mice were intraperitoneally injected with CY (50,100 or 200 mg/kg) on day 0,

Table 1. Changes of hemolytic plaque froming cells and antibody titers after immunization in ICR mice

Immune response	Days after immunization			
	2	4	6	8
PFC/10^6 spleen cells	1.61± 0.96	85.94± 44.00	20.29± 12.19	1.75± 1.92
PFC/spleen ($\times 10^2$)	3.19± 1.79	243.03± 89.32	41.80± 22.24	1.92± 2.04
HA[a] ($\log_2\times$)	0	3.7± 0.9	5.0± 2.1	0.7± 1.1
HY[b] ($\log_2\times$)	0	4.2± 1.2	5.0± 2.1	1.7± 0.7

Mice were immunized with 1×10^8 SRBC into the tail vein on day 0. PFC were determined by Chunningham's method. Each value represents mean± S.D. for 6 mice. [a] Hemagglutinin titer. [b] Hemolysin titer.

Table 2. Effects of cyclophosphamide on body and immunoorgan weights in ICR mice

Group	Dose (mg/kg)	Body weight (g)	% of Body weight		
			Liver	Spleen	Thymus
Normal[a]	–	30.2± 2.2	6.65± 0.35	0.65± 0.03	0.22± 0.05
Control	–	30.0± 2.0	7.00± 0.40	0.72± 0.08◆	0.23± 0.03
Cyclophosphamide	50	28.0± 1.5*	6.67± 0.50	0.56± 0.06**	0.18± 0.04*
	100	26.3± 1.4**	6.59± 0.76	0.38± 0.10**	0.13± 0.03**
	200	25.6± 1.5**	6.35± 0.40	0.27± 0.06**	0.09± 0.02**

Cyclophosphamide was intraperitoneally injected on day 1, at the next day mice were immunized with 1×10^8 SRBC into the tail vein, and body and organ weights were measured on day 4. Each value represents mean± S.D. for 5 mice. The significance was evaluated by Student's t-test. Significant difference from the control, *; $p<0.05$, **; $p<0.005$, and from the normal, ◆; $p<0.05$. [a] Mice were not immunized with SRBC.

at the next day immunized with 1×10^8 SRBC and immune responses were determined on day 4 (i.e. after 4 days of CY administration). As shown in Table 2, CY inhibited increases in body, spleen and thymus weights but liver weights tended to be decreased dose-dependently. The ratio of spleen to body weight in control group (immunized with SRBC) was significantly increased in comparison with that in normal group (not immunized with SRBC). The marked and dose-dependent decreases in WBC count and Hb content by CY are shown in Fig. 3. As shown in Table 3, CY depressed severely antibody titers and the number of PFC. At a dose of 50 mg/kg, CY showed to suppress antibody titers to 0 or 1 and to decrease total spleen cellurality by 40% and the number of PFC by 50%. Antibody titers and the number of PFC were not detectable at a dose of either 100 or 200 mg/kg. These results might provide optimal experimental model. The detailed experimental schedule used to assess effects of ginseng components on immunotoxicity of CY was described in Materials and Methods.

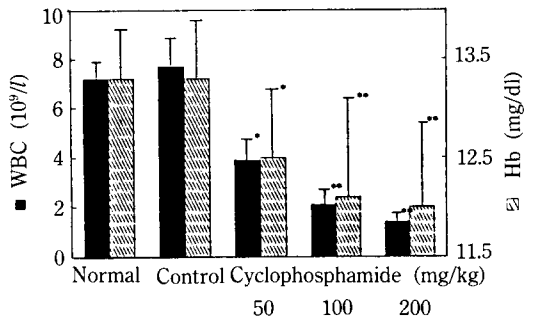


Fig. 3. Effects of cyclophosphamide on white blood cells and hemoglobin in ICR mice. Cyclophosphamide was intraperitoneally injected on day 0, at the next day mice were immunized with 1×10^8 SRBC into the tail vein, and WBC and Hb were measured on day 4. Each bar represents mean± S.D. for 6 mice. Significantly from the control, *; $p<0.05$, **; $p<0.005$.

2. Effects of Ginseng Components on Immunotoxicity of CY

As shown in Table 4, FO increased markedly the ratio of spleen to body weight in mice treated with

Table 3. Effect of cyclophosphamide on antibody titers and hemolytic plaque forming cells in ICR mice

Group	Dose (mg/kg)	HA[a] ($log_2\times$)	HY[b] ($log_2\times$)	No. of total spleen cell ($\times10^8$)	PFC/10^6 spleen cells	PFC/spleen ($\times10^2$)
Normal[c]	–	–	–	2.53± 0.40	–	–
Control	–	3.8± 0.8	3.2± 0.6	2.63± 0.42	42.33± 15.60	90.09± 25.01
Cyclophos-	50	± [d]	± [d]	1.57± 0.48**	21.15± 8.10**	33.20± 12.50**
phamide	100	0**	0**	1.06± 0.27**	1.37± 0.80**	1.74± 0.91**
	200	0**	0**	0.39± 0.04**	0**	0**

Cyclophosphamide was intraperitoneally injected on day 0, at the next day mice were immunized with 1×10^8 SRBC into the tail vein, and on day 4 antibody titers and PFC measured by Cunningham's method were determined. Each value represents mean± S.D. for 5 mice. The significance was evaluated by Student's t-test. Significant difference from the control, *; $p<0.05$, **; $p<0.005$. [a] Hemagglutinin titer. [b] Hemolysin titer. [c] Mice were not immunized with SRBC. [d] Antbody titers were 1 or 0.

Table 4. Effects of ginseng components on body and organ weights in ICR mice treated with or without cyclophosphamide

Group	Cyclophosphamide (50 mg/kg)	Body weight (g)	% of Body weight		
			Liver	Spleen	Thymus
Control	–	29.4± 1.8	6.93± 0.85	0.69± 0.08	0.19± 0.05
	+	27.5± 1.6◆	7.05± 0.53	0.52± 0.11◆	0.14± 0.05◆
Saponin	–	29.3± 2.0	7.01± 0.82	0.68± 0.08	0.20± 0.03
(50 mg/kg)	+	27.1± 2.0	7.01± 0.55	0.54± 0.07	0.14± 0.07
FO	–	27.4± 1.5◆	7.49± 0.80	1.13± 0.06◆◆	0.21± 0.05
(300 mg/kg)	+	26.3± 1.2	7.47± 0.68	0.67± 0.08*	0.15± 0.03
Panaxytriol	–	27.5± 1.5◆	6.88± 1.09	0.68± 0.15	0.18± 0.06
(20 mg/kg)	+	26.5± 1.5	7.04± 0.93	0.61± 0.13	0.13± 0.06

Ginseng components were intraperitoneally administered from day 1 to day 6. On day 3, mice were intraperitoneally injected with cyclophosphamide and immunized with 1×10^8 SRBC into tail vein on day 4. Body and organ weights were measured on day 7. Each value represents mean± S.D. for 7 or 8 mice. The significance was evaluated by Student's t-test. Significant difference from cyclophosphamide-untreated control, ◆; $p<0.05$, ◆◆; $p<0.005$, and from cyclophosphamide-treated control, *; $p<0.05$.

Table 5. Effect of ginseng components on white blood cells, hemoglobin and antibody titers in ICR mice treated with or without cyclophosphamide.

Group	Cyclophosphamide (50 mg/kg)	WBC($\times10^9$)	Hb(mg/dl)	HA[a] (log_2X)	HY[b] (log_2X)
Control	–	6.4± 1.3	13.5± 0.7	3.2± 1.1	3.5± 0.7
	+	2.6± 0.9◆◆	12.2± 0.8◆◆	± [c]	± [c]
Saponin	–	6.8± 2.0	14.4± 0.5◆	3.7± 0.6	4.1± 0.8
(50 mg/kg)	+	2.7± 0.9	13.0± 1.1	± [c]	± [c]
FO	–	9.7± 2.3◆◆	13.8± 0.9	3.5± 0.6	3.2± 0.8
(300 mg/kg)	+	8.4± 2.8**	12.4± 0.5	± [c]	± [c]
Panaxytriol	–	7.6± 2.6	13.8± 1.0	3.0± 0.7	3.0± 0.5
(20 mg/kg)	+	3.5± 0.8*	12.6± 1.3	± [c]	± [c]

Ginseng components were intraperitoneally administered from day 1 to day 6. On day 3, mice were intraperitoneally injected with cyclophosphamide and immunized with 1×10^8 SRBC into tail vein on day 4. WBC and Hb measured by coultor counter and antibody titers were determined on day 7. Each value represents mean± S.D. for 7 or 8 mice. The significance was evalulated by Student's t-test. Significant difference from cyclophosphamide untreated control, ◆; $p<0.05$, ◆◆; $p<0.005$, and from cyclophosphamide treated control, *; $p<0.05$, **; $p<0.005$. a) Hemagglutinin titer. b) Hemolysin titer. c) Antibody titers were 0 or 1.

Table 6. Effects of ginseng components on hemolytic plaque forming cells in ICR mice treated with or without cyclophosphamide

Group	Cyclophosphamide (50 mg/kg)	No. of total spleen cells ($\times 10^8$)	PFC/10^6 spleen cells	PFC/spleen ($\times 10^2$)
Control	−	2.42± 0.68	32.61± 10.15	70.74± 7.53
	+	1.48± 0.48◆	14.67± 4.83◆◆	16.27± 7.25◆◆
Saponin	−	2.59± 0.38	34.03± 9.89	85.07± 18.01◆
(50 mg/kg)	+	1.50± 0.38	16.31± 7.26	19.18± 8.07
FO	−	4.86± 1.00◆◆	58.70± 12.10◆◆	212.22± 23.48◆◆
(300 mg/kg)	+	2.15± 0.62*	26.41± 8.43**	30.42± 11.96*
Panaxytriol	−	2.32± 0.47	27.39± 6.15	65.79± 12.96
(20 mg/kg)	+	1.50± 0.59	15.12± 8.43	20.51± 8.03

Ginseng components were intraperitoneally administered from day 1 to day 6. On day 3, mice were intraperitoneally injected with cyclophosphamide and immunized with 1×10^8 SRBC into tail vein on day 4. PFC was determined according to Cunningham's method on day 7. Each value represents mean± S.D. for 7 or 8 mice. The significance was evalulated by Student's t-test. Significant difference from cyclophosphamide-untreated control, ◆; $p<0.05$, ◆◆; $p<0.005$, and from cyclophosphamide-treated control, *; $p<0.05$, **; $p<0.005$.

or without CY but had no effect on the loss of body and thymus weights by CY treatment. Neither saponin nor panaxytriol affected the ratio of immunoorgans to body weights in both control groups. However, increase of body weights was significantly suppressed by FO and panaxytriol in normal mice.

As shown in Table 5, FO inhibited markedly leucopenia induced by CY and also significantly increased WBC count within normal count range at a given dose in normal mice. Especially, panaxytriol which is known to be cytotoxic substance prevented significantly decrease in WBC count by CY. All of ginseng components had no effect on either CY-induced impairment of Hb content or antibody titers. But saponin fraction caused a significant elevation in Hb content in normal mice.

As shown in Table 6, FO inhibited decrease in the number of total spleen cells and PFC to SRBC in CY-treated mice, and increased these variables in normal mice. No significant effects of saponin fraction or panaxytriol on the number of PFC to SRBC in CY-injected mice were observed. In normal mice, saponin increased significantly the number of PFC/spleen by 20% as compared with CY-untreated control. As reported previously,[23] panaxtriol did not affect the number of total spleen cells or PFC in normal mice.

Discussion

Cyclophosphamide(CY) has extensively been used for cancer theraphy in the clincal part. But CY has been known to possess severe side effects, such as myelosuppression and immunosuppresion. These side effects become the limited factors for cancer theraphy. The effects of CY on immune system are to be mediate by its metabolites rather than the parent compound.[2-4] It is firstly converted by hepatic cytochrome P-450 into a hydroperoxide which is subsquently reduced to the corresponding alcohol, 4-hydroxy-CY which rearranges to aldophosphoramide. Aldophosphoramide undergoes β-elimination to form acrolein and the alkylating compound, phosphoramide mustard (PAM). Two reactive metabolites of CY, acrolein and PAM are known to primarily bind to sulfhydryl groups and DNA, respectively. The binding of PAM to DNA is purported to mediate the antitumor and immunosuppressive effects of CY by inhibiting cell proliferation. On the other hand, the binding of acrolein to sulfhydryl groups of cellular macromolecules may mediate the CY inhibition of Ts generation.

Treatment modalities which restore and/or counteract the detrimental side effects of CY have been investigated. BCG, *Corynebacterium pavum* and maleic anhydride divinyl ether copolymer (MVE-2) have been utilized for nonspecific immunostimulation with chemotheraphy in cancer patients.[24] Also, the fungal glucans well known to have antitumor

activity by activation of immune system, lentinan, schizophyllan and krestin (PSK) are available for cancer immunotheraphy.[24-26] It has been reported that Korean ginseng reduced the side effects of anticancer agents, such as 5-fluorouracil, mitomycin C, vinblastin and CY.[14-16] Especially ginseng showed to significantly inhibit leukopenia induced by anticancer agents.

In the present study, it was observed that crude ginseng polysaccharide fraction (FO) had inhibitory effects against decreases by CY in spleen weight and the number of PFC to SRBC as well as WBC count. Also these parameters were increased by FO in normal mice. However, neither saponin fraction nor panaxytriol had effect on impaired the number of PFC and antibody titers by CY.

In recent years, polysaccharides have emerged as an important class of bioactive natural products. Also, ginseng polysaccharides have been reported to have immunomodulating,[19,27-29] antitumor,[19,30,31] hypoglycemic[32-35] and anti-complementary activity,[36] and to inhibit toxohormone-L-induced lipolysis.[37] In the previous study,[19] FO showed various immunomodulating effects (mitogenic, polyclonal B-cell activating, reticuloendotherial system-activating and antitumor activities). The total polysaccharide fraction[30] and partially purified polysaccharide, PSG A[31] from ginseng roots have been reported to increase the number of circulating WBC in normal mice and to inhibit decrease in WBC count induced by CY. Our observation that FO increased WBC counts in both normal and CY-treated mice is consistent with results reported by other investigators.

In this study, saponin fraction showed significantly to increase the number of PFC/spleen in normal mice Also, Jie *et al.*[38] reported that oral administration of ginseng saponin increased the number of PFC/spleen in a dose-dependent manner. The finding that saponin increased significantly Hb content in normal mice might be related to the reports that ginseng extract stimulated hematopoietic function.[39,40] Panaxytriol prevented significantly leukopenia induced by CY, suggesting that panaxytriol might not cause leukopenia or lymphocytopenia, unlike other directly cytocidal anticancer agents.

Finally, throughout the immunosuppresive experimental model using CY, it is suggested that ginseng polysaccharide fraction might protect or modulate imunotoxicity of CY.

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