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Effects of Polysaccharide Ginsan from *Panax ginseng* on Liver Function

Jie-Young Song, Medea Akhalaia[1], Alexander Platonov[2], Hyung-Doo Kim, In-Sung Jung, Young-Soo Han, and Yeon-Sook Yun

Laboratory of Immunology, Korea Institute of Radiological & Medical Sciences, KAERI, 215-4, Gongneung-dong, Nowon-ku, Seoul 139-706, Korea, [1]Laboratory of Radiation Biophysics, Department of Biophysics, Moscow State University, Vorobiovi Gory, 119899, Moscow, Russia, and [2]Department of Radioecology, Ecological Faculty, Peoples Friendship University of Russia, Podolskoe shosse, 8/5, 113093, Moscow, Russia

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Ginsan, a polysaccharide isolated from *Panax ginseng*, has been shown to be a potent immunomodulator, producing a variety of cytokines such as TNF-α, IL-1β, IL-2, IL-6, IL-12, IFN-γ and GM-CSF, and stimulating lymphoid cells to proliferate. In the present study, we analyzed some immune functions 1^{st}-5^{th} days after ginsan i.p. injection, including the level of non-protein thiols (NPSH) as antioxidants, heme oxygenase (HO) activity as a marker of oxidative stress, zoxazolamine-induced paralysis time and level of hepatic cytochrome P-450 (CYP450) as indices of drug metabolism system, and activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, and albumin level as indicators of hepatotoxicity. Ginsan in the dose of 100 mg/kg caused marked elevation (1.7~2 fold) of HO activity, decrease of total CYP450 level (by 20-34%), and prolongation of zoxazolamine-induced paralysis time (by 65-70%), and showed some differences between male and female mice. Ginsan treatment did not seem to cause hepatic injury, since serum AST, ALT, and ALP activities and levels of total bilirubin and albumin were not changed.

Key words: Ginsan, Polysaccharide, Non-protein thiols, Cytochrome P450, Heme oxygenase

INTRODUCTION

Herbal medicines derived from plant extracts are widely used to treat a broad range of clinical diseases, without exact pharmacochemical and pharmacodynamical information.

Ginsan, polysaccharide isolated from the root of *Panax ginseng* C.A. Meyer, has been shown to be a potent immunomodulator, producing several cytokines (TNF-α, IL-1β, IL-2, IL-6, IL-12, IFN-γ, GM-CSF) and stimulating lymphoid cells to proliferate (Lee *et al*., 1997; Kim *et al*., 1998; Song *et al*., 2002; Shin *et al*., 2002; Song *et al*., 2003). In addition, ginsan possesses marked radioprotective and anti-septic properties (Lim *et al*., 2002; Song *et al*., 2003). However, very little information is available to date about mechanism of its immunomodulator activity, its effects on the function of liver, which is the main organ to metabolize/detoxify xenobiotics, and maintenance of antioxidant status of whole organism.

The state of antioxidant systems is a very important indicator of liver function. Among non-enzymatic antioxidant factors, the most powerful role of liver is played by reduced glutathione (GSH), the main intracellular non-protein thiol (NPSH), which has a direct radical-scavenging ability as well as an essential cofactor of the several antioxidant and detoxifying enzyme systems (Rahman and MacNee, 2000; Moran *et al*., 2001; Dickinson and Forman, 2002). Among enzymatic antioxidant factors, microsomal heme oxygenase (HO) (Maines, 1988; 1997) is considered to be a very important antioxidant enzyme, because it transforms free heme (a strong prooxidant) into biliverdin (quickly converted into a powerful antioxidant bilirubin), ferrous iron (rapidly induces cytoprotective iron-sequestering protein ferritin), and carbon monoxide (an active participant in signal transduction and possesses potent anti-inflammatory properties) (Applegate *et al*., 1991; Ryter and Tyrrell, 2000; Clark *et al*., 2000; Otterbein *et al*., 2003).

Correspondence to: Yeon-Sook Yun, Laboratory of Immunology, Korea Institute of Radiological & Medical Sciences, KAERI, 215-4, Gongneung-dong, Nowon-ku, Seoul 139-706, Korea
Tel: 82-2-970-1308, Fax: 82-2-970-2402
E-mail: immu@kcch.re.kr

The ability of liver to metabolize a variety of both endobiotics and xenobiotics is considered as one of the most important liver functions. The first stage of this metabolism is carried out by the microsomal mixed-function oxidase system which has a cytochrome P450 (CYP450) as a main enzyme (Nebert and Gonzalez, 1987; Schuetz, 2001; Nebert and Russell, 2002). This system, however, is responsible not only for detoxification of some compounds, but also for activation of some procarcinogens to carcinogens. Plant-derived preparations have been reported to have some unwanted side effects on CYP450-dependent oxidative metabolism that can cause serious alterations in drug effectiveness and toxicity or enhancement of procarcinogens activation (Ryu and Chung, 2003).

The present study was undertaken to evaluate the effect of ginsan on antioxidant status of liver and CYP450 function, and estimate its hepatotoxicity in male and female C57BL/6 mice. We analyzed the parameters including, hepatic NPSH and CYP450 levels, HO activity, zoxazolamine-induced paralysis time, and serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin and albumin levels as biomarkers of hepatotoxicity.

Results showed that ginsan treatment elevated HO activity and inhibited CYP450 activity, having some differences between male and female animals. However, hepatic injuries after ginsan treatment were not observed.

MATERIALS AND METHODS

Chemicals

β-NADPH, BSA, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), EDTA-Na_2, Tris, TCA, and olive oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, hemin chloride, and chloroform from ICN Biomedicals Inc. (Aurora, OH, USA), 2-amino-5-chlorobenzoxazole (zoxazolamine) from Oakwood Products Inc. (West Columbia, SC, USA), sodium hydrosulfite (sodium dithionite) from Aldrich Chemical Co. (Milwaukee, WI, USA).

Animals

Six- to 8-week-old female and male C57BL/6 mice were purchased from Daehan Biolink (Korea) and acclimatized for at least 7 days after their arrival before the initiation of any experimental procedure. The mice were housed in conventional conditions with 12-h light-dark cycle, temperature of 22 ± 2 °C, relative humidity of 60%, standard pelleted diet (NIH-7 open formula) and tap water *ad libitum*. The animal care, handling, and experimental procedures were conducted in accordance with the guidelines approved by the Animal Care and Use Committee of the Korea Institute of Radiological & Medical Sciences (KIRAMS).

Ginsan preparation and treatment

The polysaccharide ginsan was purified from the ethanol-insoluble fraction of *Panax ginseng* water extract as described previously (Lee *et al.*, 1997; Song *et al.*, 2003). We have self-sufficient ginsan which was prepared under the well-controlled process and exhibited high activity, and always used new preparations after confirming the chemical and biological activity such as gel permeability chromatography (GPC) patterns, contents of carbohydrates, LAK cell generation, production of nitric oxides and proliferation of splenocytes comparing to the standard ginsan. Ginsan was dissolved in phosphate-buffered saline (PBS; pH 7.4), filtered through 0.25-μm Millipore membranes, and administered intraperitoneally in a volume of 0.2 mL/mouse. Control animals were given 0.2 mL of PBS.

Assay of NPSH content

NPSH content in liver was determined with DTNB by the method of Sedlak and Lindsay (Sedlak and Lindsay, 1968). Briefly, immediately after sacrifice of mice by cervical dislocation, 50-90 mg of liver were quickly harvested, minced with scissors, and homogenized in a mixture of 2 mL 10% TCA and 2 mL 0.02 M EDTA-Na_2 on ice by a Tissue-Tearor (BioSpec Products, Inc., USA). The homogenates were centrifuged at 3500 g for 20 min at 4 °C. Then, 0.5 mL of the supernatant was mixed with 1 mL 0.4 M Tris-HCl buffer (pH 8.9) containing 0.02 M EDTA-Na_2, and 25 μL of 0.01 M DTNB in methanol were added to each sample. Optical density (OD) was measured at 412 nm in 5 min. Blank sample contained all the above-mentioned components, except the supernatant. NPSH contents were calculated using an extinction coefficient (ε_{412}) of 13.1 mM $^{-1}$cm^{-1} and expressed in micromoles per gram of wet tissue weight.

HO activity assay

HO activity in liver was determined by the method described earlier (Maines and Kappas, 1978; Ryter *et al.*, 2000). Briefly, immediately after sacrifice of mice, livers were quickly harvested, minced with scissors, and homogenized in 4 volumes of 100 mM K-phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$ ($MgCl_2$-phosphate buffer) on ice. The homogenates were centrifuged at 1500 g for 10 min at 4 °C, and the supernatants obtained were further centrifuged at 15,000 g for 15 min at 4 °C. The 15,000 g supernatants were kept at 4 °C and used for determination of HO activity not later than 1-1.5 h after preparation. The reaction mixture for determination of HO activity included $MgCl_2$-phosphate buffer, 5 mg of the 15,000 g supernatant protein, 0,5 mg of protein of liver 105,000 g supernatant as a source of biliverdin reductase (see below for pre-

paration), 2 mM glucose 6-phosphate, 1 U of glucose-6-phosphate dehydrogenase, 25 μM hemin, and 1 mM NADPH in a final volume of 500 μL. Reaction was initiated by the addition of NADPH, and the reaction mixtures were incubated for 10 min in a water bath at 37 °C in the dark. To terminate the reaction and extract produced bilirubin, 500 μL of chloroform were added to each sample, the samples were vortexed thoroughly for 30 s, and centrifuged at 15,000 g for 10 min. The lower chloroform layer was used for determination of $\Delta OD_{464-530}$ by UV/Visible spectrophotometer Ultrospec 3100 pro (Biochrom, Ltd., England). Bilirubin concentrations were calculated using the extinction coefficient ($\varepsilon_{464-530}$) of 40 mM^{-1} cm^{-1}. HO activity was expressed in picomoles of bilirubin produced per milligram of protein per hour.

As a source of biliverdin reductase, liver 105,000 g supernatant was used (Maines and Kappas, 1978). To prepare the supernatant, livers of overnight-fasted mice were perfused *in situ* with cold (4 °C) 1.15% KCl, harvested, minced with scissors, and homogenized in 4 volumes of $MgCl_2$-phosphate buffer on ice. The homogenates were centrifuged at 1500 g for 10 min at 4 °C, and the supernatants obtained were successively centrifuged at 15,000 g for 15 min at 4 °C and at 105,000 g for 1.5 h at 4 °C. The 105,000 g supernatant was diluted to 5 mg of protein/ml with $MgCl_2$-phosphate buffer and stored at 20 °C. Protein concentration was determined by the method described earlier (Schacterle and Pollack, 1973) with BSA as a standard.

Determination of hepatic CYP450 level

The mice were sacrificed by cervical dislocation, and their livers were quickly removed and homogenized in 4 volumes of 100 mM K-phosphate buffer (pH 7.4) on ice. The homogenates were subjected to successive differential centrifugation (at 1500 g for 10 min, at 12,000 g for 15 min, and at 105,000 g for 60 min) at 4 °C to obtain the pellets of liver microsomal fraction. The microsomal pellets were resuspended in 100 mM K-phosphate buffer at 1 mg/mL protein concentration. The level of CYP450 was spectrophotometrically determined in microsomal suspension after its reduction by sodium dithionite and binding with carbon monoxide as described previously (Schoene *et al.*, 1972). Level of CYP450 was calculated using an extinction coefficient ($\varepsilon_{450-500}$) of 91 mM^{-1} cm^{-1} and expressed in nanomoles per milligram of protein.

Determination of zoxazolamine-induced paralysis time

Zoxazolamine paralysis was induced by i.p. injection of zoxazolamine at dose of 125 mg/kg (in olive oil, 0.2 mL/mouse. The duration of paralysis was defined as the time interval between loss and restoration of the righting reflex.

Serum markers of hepatic injury

Hepatic injury was assessed by ALT, AST, and ALP activities, and also total bilirubin and albumin levels in sera which were measured by the Vitros 950 analyzer (Johnson and Johnson, Ortho-Clinical Diagnostics, Inc., Rochester, NY, USA).

Statistical analysis

All statistical procedures were performed using the GraphPad Prism version 3.02 software (San Diego, CA, USA). Data are expressed as means ± SEM. Each test group consisted of at least 5 animals. Normality of data distribution was checked with Kolmogrov-Smirnov test. For comparison between two groups, two-tailed unpaired Students *t*-test was used. Equality of variance was estimated with F-test and data that failed to pass F-test were analyzed using *t*-test with Welchs correction. More than two groups were compared by one-way analysis of the variance (ANOVA) followed by post-hoc Bonferronis multiple comparison test. Differences among the groups were considered to be statistically significant at $P < 0.05$.

RESULTS

Effect of ginsan on the hepatic NPSH level

The effects of ginsan on the hepatic NPSH level of female and male mice are presented in Fig. 1. The dose of ginsan used in these experiments was 100 mg/kg, because this dose was previously shown to be optimal for excellent radioprotective effect with marked improvement of some hematopoietic indices on the 5th day after irradiation (Song *et al.*, 2003). As shown in Fig 1A, ginsan significantly increased the hepatic NPSH level on the 5th day in female mice (to 119.3% of control), but not in males (Fig. 1B). No significant changes were observed on the 1st day after the injection in both female and male mice.

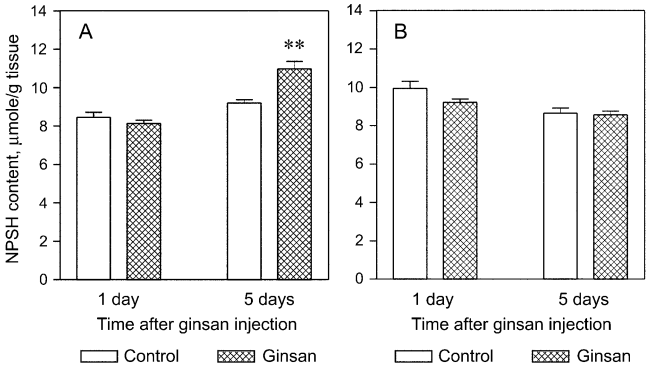

Fig. 1. Effects of ginsan injection (100 mg/kg, i.p.) on NPSH level in liver of female (A) and male (B) C57BL/6 mice. Each test group was compared with own control group. All data are presented as means ± SEM of 5 mice. $^{**}P < 0.01$, compared with corresponding control group.

However, the treatment with higher doses (5- and 10-fold) of ginsan significantly decreased the hepatic NPSH level on the 1st day after the injection (data not shown).

Effect of ginsan on the hepatic HO activity

The effects of ginsan injection on the hepatic HO activity are presented in Fig. 2. The sharp elevation of hepatic HO activity was demonstrated in both female (to 168.6% of the control) (Fig. 2A) and male (to 197.5% of the control) (Fig. 2B) mice. This elevation of HO activity seemed to be short-term event, since it already returned to the control level on the 2nd day in both sexes and no changes were observed until the 5th day.

Effect of ginsan on the duration of zoxazolamine-induced paralysis

In order to determine the action of ginsan on hepatic drug metabolism, the zoxazolamine paralysis time as an *in vivo* measure of hepatic CYP1A activity was investigated in female and male mice after ginsan pretreatment. As shown in Fig. 3, zoxazolamine-induced paralysis time was markedly prolonged on the 1st day after ginsan injection in both female (to 169.3% of control) and male (to 165.5% of control) mice (Fig. 3A and 3B). On the 5th day after ginsan injection, the duration of zoxazolamine-induced paralysis slightly decreased however, stayed on the increased level in female mice (140.0% of control), whereas it completely returned to normal level in males.

Effect of ginsan on the hepatic CYP450 level

As shown in Fig. 4, ginsan treatment decreased total CYP450 level in liver microsomal fraction on the 1st and 5th days both in female and male mice. In female mice, hepatic CYP450 level was diminished to 79.6% of the control on the 1st day and lasted until the 5th day after ginsan injection (Fig. 4A). In male mice, the level decreased to 66.2% of the control on the 1st day, but partly returned to 87.1% of the control on the 5th day (Fig. 4B), suggesting that the response to ginsan was stronger in males than in females.

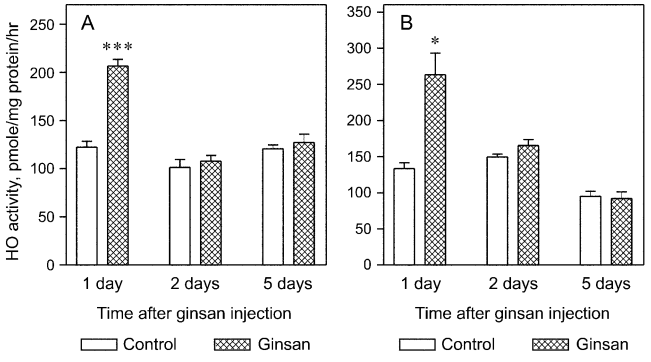

Fig. 2. Effects of ginsan injection (100 mg/kg, i.p.) on HO activity in liver of female (A) and male (B) C57BL/6 mice. Each test group was compared with own control group. All data are presented as means ± SEM of 5 mice. *P < 0.05, ***P < 0.001, compared with corresponding control group.

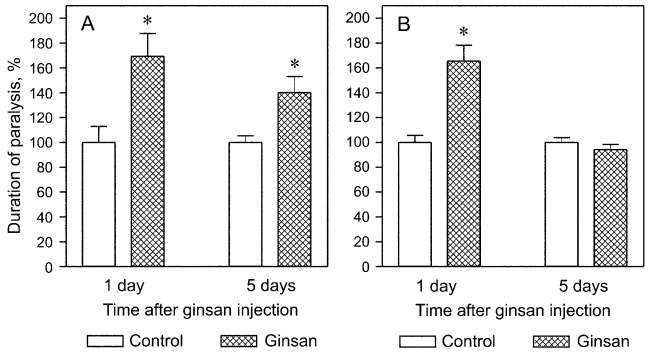

Fig. 3. Effects of ginsan injection (100 mg/kg, i.p.) on zoxazolamine-induced paralysis time in female (A) and male (B) C57BL/6 mice. Each test group was compared with own control group. All data are presented as means ± SEM of 5-6 mice. Average duration of zoxazolamine-induced paralysis was 54 min in females and 36 min in males. *P < 0.05, compared with corresponding control group.

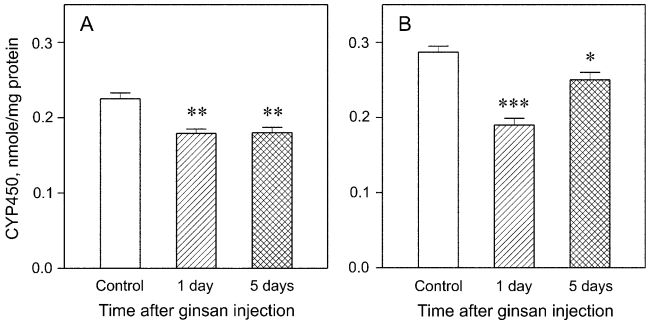

Fig. 4. Effects of ginsan injection (100 mg/kg, i.p.) on CYP450 level in liver of female (A) and male (B) C57BL/6 mice. All data are presented as means ± SEM of 5-6 mice. *P < 0.05, **P < 0.01, ***P < 0.001, compared with control group.

Table I. Effects of ginsan on serum biomarkers of hepatic injury

Biomarkers of hepatic injury	Control mice	Ginsan-treated mice	
		1 day	5 days
ALT activity, U/l	44.7 ± 3.0	36.5± 4.1	45 ± 2.5
AST activity, U/l	120.2 ±12.4	91.5±10.5	96 ±11.8
ALP activity, U/l	213.5 ±25.2	155 ±20.3	196 ±18.0
Total bilirubin level, mg/dL	0.28± 0.04	0.20± 0.05	0.30± 0.04
Albumin level, mg/dL	2.6 ± 0.1	2.2 ± 0.2	2.7 ± 0.3

Mice were treated with 100 mg/kg of ginsan. Control mice were given PBS. One and 5 days after treatment, hepatic injuries were determined by measuring the serum biomarkers described above. Data are presented as means±SEM of 6 mice per group.

Effect of ginsan on serum ALT, AST, ALP activities, total bilirubin and albumin levels

In order to evaluate whether ginsan induced hepatic injury, we determined serum ALT, AST, ALP activities, total bilirubin and albumin levels in mice 1 day and 5 days after ginsan injection. As shown in Table I, ginsan treatment did not result in any significant alterations of these biomarkers of hepatic injury.

DISCUSSION

Endogenous thiols have recently been considered to be extremely important components in redox-sensitive regulation of signal transduction, providing adaptive protective cellular response to different extrinsic stimuli (Moran *et al*., 2001). Special role in this regulation was attributed to NPSH, especially GSH which comprises overwhelming majority of cellular NPSH (Sedlak and Hanus, 1982). Furthermore, GSH is also an essential participant of several defense antioxidant and xenobiotics-detoxifying systems (Hayes and McLellan, 1999), and may have certain additional functions in the regulation of the immune response, cellular proliferation, and prostaglandin metabolism (Kang, 1994; Jakobsson *et al*., 1999; Aoshiba *et al*., 1999; Rahman and MacNee, 2000; Filomeni *et al*., 2002). GSH is synthesized in various tissues, however, the liver has the highest level of GSH and serves as an important source of GSH for other tissues (Locigno and Castronovo, 2001). It is well known that various prooxidants can stimulate GSH synthesis after its initial depletion, providing a protective/adaptive mechanism against oxidative stress (Rahman and MacNee, 2000). In the present study, the elevation of hepatic NPSH was observed in female mice on the 5th day after optimal dose of 100 mg/kg ginsan treatment, whereas 5- and 10-fold higher doses of ginsan resulted in marked depletion of NPSH in the 1st day after injection (unshown data). Moreover, the hepatic HO activity was markedly elevated on the 1st day after ginsan injection in both female and male mice. It is known that one of two main isoforms of HO, namely inducible HO-1 form, is identical to the 32-kDa stress (heat shock) protein (HSP32) and serve as a sensitive marker of oxidative stress (Taketani *et al*., 1989; Keyse and Tyrrell, 1989). HO-1 may be induced by multiple chemical and physical agents, such as heme, various heavy metals, LPS, hormones, phorbol esters, sodium arsenite, H_2O_2, sulfhydryl reagents, cytokines, UVA radiation, hyperoxia, and hyperthermia which generate reactive oxygen species (ROS) (Maines, 1997; Ryter and Tyrrell, 2000; Otterbein and Choi, 2000). Induction of HO-1 is considered as a general adaptive response to oxidant stress and plays an important role in antioxidant defense (Keyse and Tyrrell, 1989; Applegate *et al*., 1991). In line with above results, we already showed that ginsan activated macrophages to produce nitric oxides, hydrogen peroxide and several cytokines, which are front line effector molecules exerting strong cytotoxic activities against foreign agents (Shin *et al*., 2002). Thus, our data on hepatic NPSH level and HO activity together demonstrate that ginsan might act as a prooxidant through indirect activation of macrophages, and that its protective effects at certain doses may possibly be due to induction of antioxidant defense systems.

To elucidate whether ginsan can interfere with hepatic CYP450-dependent mixed-function oxidase system, we performed zoxazolamine paralysis test and measured total CYP450 level in liver. Zoxazolamine is a potent muscle relaxant which is inactivated mainly by the hepatic CYP1A2 and extrahepatic CYP1A1 enzymes, and the duration of zoxazolamine-induced paralysis is an *in vivo* measure of the CYP1A capacity (Sasaki, 1994; Liang *et al*., 1996; Dertinger *et al*., 2001). The CYP1A1/2 enzymes are of particular interest, because of their capacity not only to detoxify numerous compounds but also to activate some procarcinogens, including polycyclic aromatic hydrocarbons. Therefore, the high level of CYP1A1/2 is considered to be linked with higher cancer risk (Dertinger *et al*., 2001; Bao *et al*., 2002; Ryu and Chung, 2003). In the present study, markedly prolonged the zoxazolamine paralysis time in both female and male mice indicates the decline of the CYP1A level. The total of CYP450 level also diminished at the same time point, thus further confirming the data obtained by zoxazolamine paralysis test. The above results, therefore, led us to conclude that ginsan treatment suppresses procarcinogen-activating CYP1A form, and it may contribute to anticarcinogenic action. Some plant medicines or dietary supplements have been shown to possess the similar suppressive effect on the hepatic CYP1A capacity (Liu *et al*., 1995; Jeong and Lee, 1999; Thapliyal and Maru, 2001; Ueng *et al*., 2003) while others are inductive (Ueng *et al*., 2001; Ryu and Chung, 2003). Depletion of CYP450 level after ginsan treatment might have occurred through i) action of some ginsan-induced cytokines including TNF-α, IL-1β, IL-2, IL-6, and IFN-γ (Lee *et al*., 1997; Song *et al*., 2002) that have suppressive effect (Abdel-Razzak *et al*., 1993; Renton, 2000), ii) direct destructive action of ROS on CYP450 (Jeffery *et al*., 1977; Karuzina and Archakov, 1994), or iii) increased HO activity that can limit the availability of heme for CYP450 synthesis as well as direct attack on heme of CYP450 (Maines, 1988; 1997). On the other hand, the excessive pool of free heme originated from labilized hepatic hemoproteins (especially CYP450) can induce HO (Maines, 1988; 1997). Indeed, in the present study ginsan was found to depress CYP450 level and elevate HO activity.

The most frequent side effect of medicines is a serious liver injury. Therefore, we evaluated whether ginsan induced

hepatotoxicity through five serological indices, namely ALT, AST, ALP activities, and total bilirubin and albumin levels. It is known that the rises of AST, ALT and ALP activities in serum are attributable to the damaged structural integrity of the liver, because these enzymes have intracellular localization and are released from liver into circulation after cell damage. High total bilirubin level and decreased serum albumin level can also indicate advanced liver injury. Therefore these serum markers are widely used for estimation of hepatotoxicity of different agents (Seubert *et al*., 2002; Kumar Rajagopal *et al*., 2003; Ozbek *et al*., 2003). Our data demonstrated that ginsan in the dose of 100 mg/kg did not alter serum ALT, AST, ALP activities, and total bilirubin and albumin levels indicating no hepatotoxic action of ginsan itself in mice.

To sum up, we demonstrated in the present study that ginsan might have modulating effects on antioxidant status and drug metabolism of liver in female and male mice. These effects indicate that ginsan acts as an indirect prooxidant and its action is not too severe to cause any hepatic injury, but strong enough to induce antioxidant defence systems. Depression of CYP450-dependent enzyme system by ginsan should be taken into account, when other drugs and xenobiotics are concurrently taken. Study on detailed action mechanisms of ginsan on liver function as well as simultaneous application with other drugs should further be investigated.

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