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Effects of Ginsenosides on $GABA_A$ Receptor Channels Expressed in Xenopus Oocytes

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Ginsenosides, major active ingredients of *Panax* ginseng, are known to regulate excitatory ligand-gated ion channel activity such as nicotinic acetylcholine and NMDA receptor channel activity. However, it is not known whether ginsenosides affect inhibitory ligand-gated ion channel activity. We investigated the effect of ginsenosides on human recombinant $GABA_A$ receptor ($\alpha_1\beta_1\gamma_{2S}$) channel activity expressed in *Xenopus* oocytes using a two-electrode voltage-clamp technique. Among the eight individual ginsenosides examined, namely, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 and Rg_2, we found that Rc most potently enhanced the GABA-induced inward peak current (I_{GABA}). Ginsenoside Rc alone induced an inward membrane current in certain batches of oocytes expressing the $GABA_A$ receptor. The effect of ginsenoside Rc on I_{GABA} was both dose-dependent and reversible. The half-stimulatory concentration (EC_{50}) of ginsenoside Rc was 53.2 ± 12.3 μM. Both bicuculline, a $GABA_A$ receptor antagonist, and picrotoxin, a $GABA_A$ channel blocker, blocked the stimulatory effect of ginsenoside Rc on I_{GABA}. Niflumic acid (NFA) and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), both Cl^- channel blockers, attenuated the effect of ginsenoside Rc on I_{GABA}. This study suggests that ginsenosides regulated $GABA_A$ receptor expressed in *Xenopus* oocytes and implies that this regulation might be one of the pharmacological actions of *Panax* ginseng.

Key words: *Panax* ginseng, Ginsenosides, GABA, $GABA_A$ receptor, Ligand-gated ion channels, *Xenopus* oocytes

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, is a well known traditional medicine and tonic. The main molecular components responsible for the actions of ginseng are ginsenosides, which are also known as ginseng saponins. Ginsenosides have a four-ring, steroid-like structure with attached sugar moieties. About 30 different types of ginsenosides have been isolated and identified from the root of *Panax* ginseng. The ginsenosides are classified into protopanaxadiol and protopanaxatriol ginsenosides according to the positioning of sugar moieties at the carbon-3 and -6 (Attele *et al.*, 1999).

Recent reports have shown that ginsenosides might regulate ligand-gated ion channel activity. Moreover, ginsenosides are known to inhibit acetylcholine-stimulated catecholamine release in cells expressing nicotinic acetylcholine receptors, such as bovine chromaffin cells (Kudo *et al.*, 1998; Tachikawa *et al.*, 1999). In addition, Choi *et al.* (2002) and Sala *et al.* (2002) showed that protopanaxatriol ginsenosides inhibit acetylcholine-induced inward current in *Xenopus* oocytes expressing neuronal and muscle-type nicotinic acetylcholine receptors. In addition, in cultured rat hippocampal neurons, ginsenoside Rb_1 and Rg_1 were found to reduce glutamate-induced cell death (Abe *et al.*, 1994). Moreover, ginsenosides and ginsenoside Rg_3 also attenuated glutamate-induced neurodegeneration by inhibiting the overproduction of nitric oxide, the formation of malondialdehyde, and Ca^{2+} influx in rat cortical cultures and in hippocampal slices (Kim *et al.*, 1998; Kim *et al.*, 2002). Finally, Seong *et al.* (1995) showed that ginsenosides attenuate the glutamate-induced swelling of cultured rat astrocytes.

The $GABA_A$ receptor is one of a superfamily of ligand-gated ion channel receptors, which share structural similarity with nicotinic acetylcholine, 5-HT_3, and glycine receptors

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(Ortells and Lunt, 1995). The $GABA_A$ receptor is predominantly expressed in the central nervous system (Bloom and Iversen, 1971; McCabe and Wamsley, 1986), and forms a chloride-selective transmembrane channel in the post-synaptic sites of nerve terminals. Thus, the $GABA_A$ receptor is responsible for fast inhibitory synaptic transmission Macdonald and Olsen, 1994; Whiting *et al.*, 1995). Recent biochemical binding assays produced evidence that ginsenosides might regulate the $GABA_A$ receptor. For example, Kimura *et al.* (1994) showed that ginsenosides differentially regulate [^{3}H] flunitrazepam or [^{3}H] muscimol binding to the $GABA_A$ receptor in a rat brain membrane fraction. Whereas, Kim *et al.* (2001) showed that prolonged infusion with ginsenoside Rc but not with ginsenoside Rg_1 into rat brain elevates [^{3}H] muscimol binding to the $GABA_A$ receptor in a brain region-specific manner.

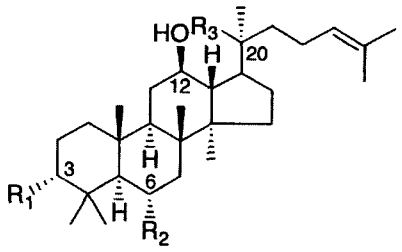
Thus, ginsenosides may regulate the $GABA_A$ receptor by affecting ligands affinity on its receptor, but there is no direct evidence the regulation of $GABA_A$ receptor channel activity by ginsenosides. In this study, we examined the effects of ginsenosides on $GABA_A$ receptor channel activity. For this study, we injected neuronal human $GABA_A$ ($\alpha_1\beta_1\gamma_{2S}$) receptor cRNAs into *Xenopus* oocytes and examined the effect of ginsenosides on the GABA-elicited inward peak currents (I_{GABA}). The reasons we used this system are as follows: (1) *Xenopus laevis* oocytes have widely been used as a tool to express membrane proteins encoded by exogenously administered cDNAs or cRNAs, including those of receptors, ion channels, and transporters (Dascal, 1987 and (2) The $GABA_A$ receptor ($\alpha_1\beta_1\gamma_{2S}$) channels expressed in *Xenopus* oocytes by injecting $GABA_A$ receptor cRNAs subunits have been well studied and characterized (Hill-Venning *et al.*, 1997). Accordingly, in this study we undertook to investigate the effect of ginsenosides on human recombinant $GABA_A$ receptor ($\alpha_1\beta_1\gamma_{2S}$) channel activity expressed in *Xenopus* oocytes using a two-electrode voltage-clamp technique. We found that the treatment of ginsenoside Rc enhanced I_{GABA} in a reversible, dose-dependent, bicuculline and picrotoxin-sensitive manner.

MATERIALS AND METHODS

Materials

Ginsenosides were kindly provided by the Korean Ginseng and Tobacco Research Institute (Taejon, Korea). Fig. 1 shows the structures of the five representative ginsenosides. The ginsenosides used in this study were dissolved in dimethyl sulfoxide (DMSO) and diluted with bath medium before use. The final DMSO concentration was less than 0.01%. Other chemical agents were obtained from Sigma (St. Louis, MO, USA).

Oocyte preparation



Ginsenoside	R_1	R_2	R3
Ginsenoside-Rb_1	-O-Glc^2-Glc	-H	-O-Glc^6-Glc
Ginsenoside-Rc	-O-Glc^2-Glc	-H	-O-Glc^6-Ara (pyr)
Ginsenoside-Re	-OH	-O-Glc^2-Rha	-O-Glc
Ginsenoside-Rf	-OH	-O-Glc^2-Glc	-OH
Ginsenoside-Rg_1	-OH	-O-Glc	-O-Glc

Fig. 1. Structure of the five representative ginsenosides

Xenopus laevis care and handling was in accordance with the guide for the *Care and Use of Laboratory Animals* published by NIH, USA. Frogs underwent surgery only twice, separated by at least 3 weeks. To isolate oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester. Oocytes were separated by treatment with collagenase, followed by gentle shaking for 2 h in $CaCl_2$-free medium containing 82.5 NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units of penicillin per ml, and 100 μg streptomycin/ml. Only stage 5 or 6 oocytes were collected and maintained at 18°C with continuous gentle shaking in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 μg gentamycin/ml. All solutions were changed daily, and experiments were performed within 2-4 days following the isolation of the oocytes.

Oocyte recording

A single oocyte was placed in a small Plexiglas net chamber (0.5 ml), which was constantly superfused with ND96 medium in the absence or presence of GABA or ginsenosides during recording. The microelectrodes were filled with 3 M KCl and had a resistance of 0.2-0.7 MΩ. Two-electrode voltage-clamp recordings were performed at room temperature using an Oocyte Clamp (OC-725C, Warner Instrument, Hamden, CT, USA) equipped with Digidata 1200A. For most of the electrophysiological experiments, the oocytes were clamped at a holding potential of -80 mV, and 300-ms voltage steps were applied from -100 to +40 mV to determine the nature of the current-voltage relationship. Linear leak currents were corrected using the leak subtraction procedure.

cRNA preparation of $GABA_A$ ($\alpha_1\beta_1\gamma_{2s}$) receptor and microinjection

cDNAs encoding human $GABA_A$ receptor subunits ($\alpha_1\beta_1\gamma_{2s}$)

were linearized with appropriate restriction enzymes, and the cRNAs were transcribed from linearized templates using an *in vitro* transcription kit (mMessage mMachine; Ambion, Austin, TX, USA) and T7 polymerase. The cRNA was dissolved in RNase-free water at a final concentration of approximately 1 μg/μl and stored at -70°C until used. Oocytes were injected with H_2O or human $GABA_A$ receptor cRNAs (5-10 ng) by using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA, USA). The injection pipette was pulled from glass capillary tubing used for recording electrodes and the tip was broken to ~20-μm-OD.

Data analysis

All values are presented as means ± S.E.M. Differences between the means of control and ginsenosides treatment data were analyzed using the unpaired Students *t* test. A *P* value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The addition of GABA (10 μM) to the bathing medium induced a large inward current (I_{GABA}) in oocytes injected with $GABA_A$ receptor subunits cRNAs, indicating that the $GABA_A$ receptor was functionally expressed (Fig. 2A). In a certain batch of oocytes expressing $GABA_A$ receptor, the treatment of ginsenoside Rc had no effect; however, another batch of oocytes expressing the $GABA_A$ receptor induced an inward current when treated with Rc at a holding potential of -80 mV (Figs. 2A and 3A). Moreover, pretreatment with ginsenoside Rc for 1 min before GABA induced a large increase of I_{GABA} in a reversible manner (Fig. 2B, n = 15 from three different frogs). We also tested the effects of other ginsenosides, namely, Rb_1, Rb_2, Rd, Re, Rf, Rg_1, or Rg_2 on I_{GABA} in oocytes expressing the $GABA_A$ receptor. As shown in Fig. 2B, Rc significantly enhanced I_{GABA}, and the order of potency in terms of I_{GABA} enhancement was Rc > Rb_2 > Rd; however, Rb_1, Re, Rf, Rg_1, and Rg_2 had no of insignificant effect on I_{GABA} (Fig. 2).

In dose-dependent experiments with ginsenoside Rc, pretreatment with Rc increased I_{GABA} in a dose-dependent manner in oocytes expressing the $GABA_A$ receptor (Fig. 3A). The EC_{50} of I_{GABA} was 53.2 ± 12.3 μM in oocytes expressing the $GABA_A$ receptor (n = 9-12 from three different frogs for each point) (Fig. 3B). In current-voltage experiments, the membrane potential was held at -80 mV and a voltage ramp was applied from -100 to +40 mV for 300 ms. In the absence of GABA, the inward current at -100 mV was <0.3 μA and the outward current at +40 mV was 0.3-0.5 μA. The addition of GABA to the bathing medium resulted in an increase of the inward current at a potential more negative than ca -20 mV. In contrast, at a potential more positive than ca -20 mV, GABA caused a large increase in the outward current. Pretreatment with Rc before GABA increased both inward and outward currents as compared with those induced by GABA treatment alone. The reversal potential was near -20 mV in GABA alone and in GABA plus Rc, which indicates that GABA induces the Cl^- current (Macdonald and Olsen, 1994). Also, pretreatment with Rc before GABA did not affect the channel property of the $GABA_A$ receptor (Fig. 4).

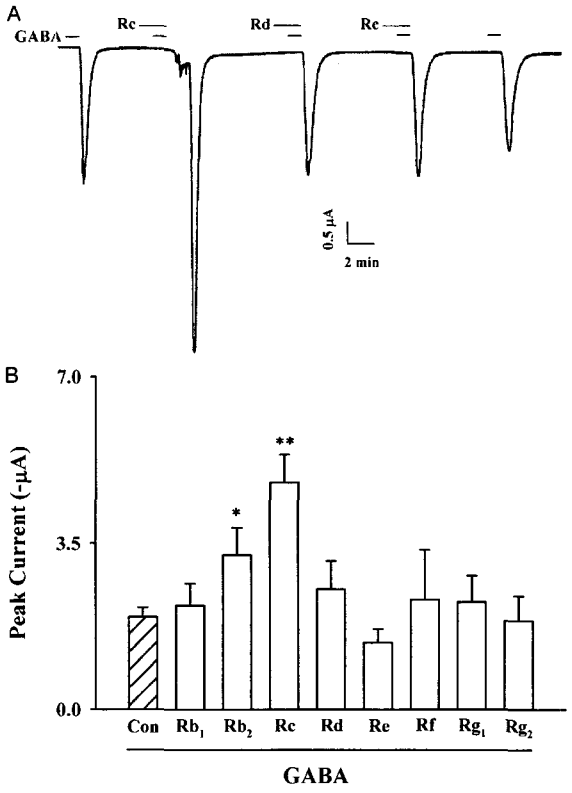


Fig. 2. Expression of $GABA_A$ receptor channel in Xenopus oocytes after $GABA_A$ receptor subunits cRNAs injection. The inward currents were recorded at a holding potential of 70 mV. **A.** GABA (GABA; 10 μM) induced a large inward current in oocytes expressing $GABA_A$ receptor. Pretreatment with ginsenoside Rc but not with ginsenoside Rd or Re (each 100 μM) before GABA treatment enhanced I_{GABA}. **B.** Histogram representing peak inward currents recorded during treatment with GABA alone (Con; 10 μM) or after pretreatment with various individual ginsenosides (i.e., Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, and Rg_2; each at 100 μM). Among the several ginsenosides examined, ginsenoside Rc shows the strongest effect on GABA-induced inward peak current. Each point represents a mean±S.E.M. (n = 8-10/group). **P*< 0.05, ***P*<0.01 compared with the GABA-alone induced inward peak current.

To further verify that the extra current elicited by pretreatment with Rc was due to GABA receptor channel current, we examined the effect of bicuculline, a $GABA_A$ receptor antagonist and picrotoxin, a $GABA_A$ channel blocker (Krishek *et al.*, 1996). As shown in Fig. 5, pretreatment with bicuculline or picrotoxin almost blocked the current elicited by the application of Rc plus GABA in a reversible manner.

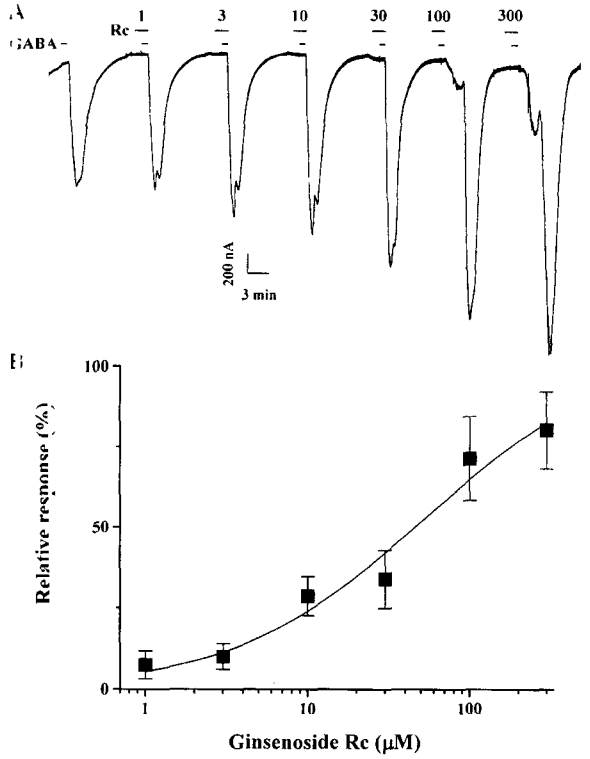


Fig. 3. Dose-dependent effect of ginsenoside Rc on I_{GABA}. A. The trace shows that ginsenoside Rc increased the currents elicited by GABA (GABA; 10 μM) in a dose-dependent manner. B. Graphic illustration of the magnitude of the inward current evoked in each experimental condition is expressed as a percentage of the current that was evoked by GABA treatment alone. Data are presented as means±S.E.M. (n = 8-10/group).

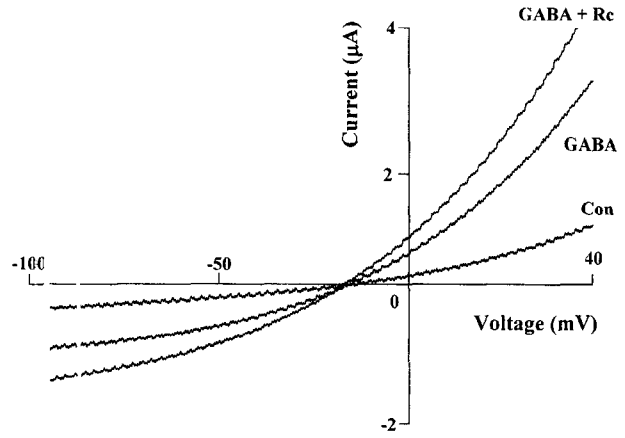


Fig. 4. This representative current-voltage relationship was obtained using voltage steps between -100 and +40 mV and a ramp protocol. Pretreatment with ginsenoside Rc (GABA + Rc, Rc; 100 μM) potentiated both the inward and outward currents induced by GABA alone (GABA; 10 μM). The reversal potential was near -20 mV.

We also tested the effect of 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS) and niflumic acid (NFA), Cl^- channel blockers, to ensure that both the inward currents elicited by GABA and the extra current elicited by Rc were Cl^- currents (Frings *et al.*, 2000). As shown in Fig. 5B, in the presence of DIDS or NFA, the stimulatory effect of Rc on I_{GABA} was substantially reduced.

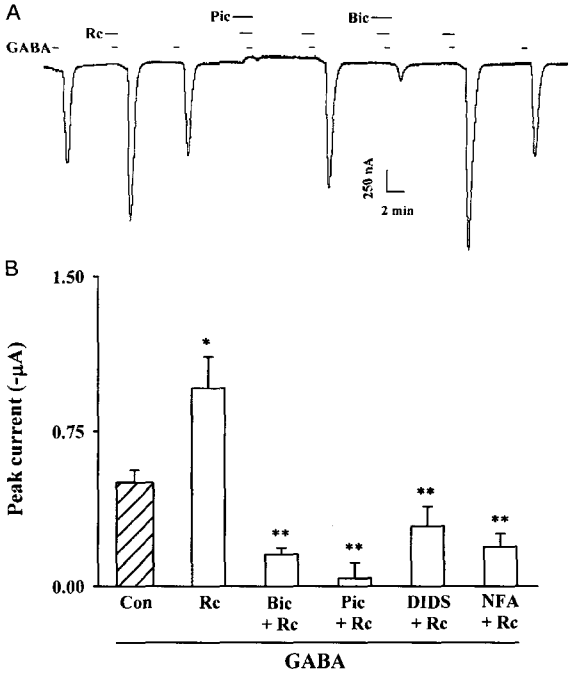


Fig. 5. Effects of the $GABA_A$ receptor antagonist, bicuculline or $GABA_A$ receptor channel blocker, picrotoxin on ginsenoside Rc-induced potentiation of I_{GABA}. **A.** Representative trace showing inward currents in the presence of GABA (GABA; 10 μM) alone, picrotoxin (Pic; 30 μM) + ginsenoside Rc (Rc; 100 μM) + GABA (GABA; 10 μM), bicuculline (Bic; 30 μM) + ginsenoside Rc (Rc; 100 μM) + GABA (GABA; 10 μM), or ginsenoside Rc + GABA. **B.** Histogram showing peak inward currents recorded during the treatment of GABA alone, ginsenoside Rc + GABA, GABA + ginsenoside Rc + bicuculline, GABA + ginsenoside Rc + picrotoxin, GABA + ginsenoside Rc + DIDS (100 μM), or GABA + ginsenoside Rc + NFA (100 μM). Each point represents the mean ± S.E.M. (n = 8-10/group). $^*P < 0.05$, $^{**}P < 0.01$ compared with the GABA-alone induced inward current.

The present study demonstrates that; (1) ginsenoside Rc enhanced I_{GABA} in reversible and dose-dependent manner in oocytes expressing the $GABA_A$ receptor; (2) the ginsenoside Rc induced enhancement of I_{GABA} was sensitive to GABA receptor antagonist and $GABA_A$ receptor channel blocker; (3) the protopanaxadiol ginsenoside Rc much more potently increased I_{GABA} than the protopanaxatriol ginsenosides such as Re, Rf, and Rg_1.

We also found that ginsenoside Rb_2, although to a lesser extent than Rc, also enhanced I_{GABA} (Fig. 2). Interestingly, ginsenoside Rc (20-S-Protopanaxadiol-3-[O-β-glucopyranosyl (1→6)-β-D-glucopyranoside]-20-[O-α-L-glucopyranosyl (1→2)-β-D-arabinopyranoside] has two glucoses at the C-3 position and one glucose and one arabinose at the C-20 position, and ginsenoside Rb_2 (20-S-Protopanaxadiol-3-

[O-β-glucopyranosyl (1→6)-β-D-glucopyranoside]-20-[O-α-L-glucopyranosyl (1→2)-β-D-glucopyranoside] has two glucoses at the C-3 position and two glucoses at the C-20 position (Fig. 1). Thus, ginsenoside Rc and Rb_2 have similar structures except that Rc has an arabinose instead of a second glucose at the C-20 position. It seems that an arabinose rather than a glucose at the C-20 position of ginsenoside Rc may play an important role in the enhancement of I_{GABA}, though this issue certainly requires further investigation.

On the other hand, these results suggest that Rc affects the $GABA_A$ receptor channel activity that mediates inhibitory neurotransmission in the central nervous system. However, it is unclear precisely how Rc acts to increase I_{GABA} in oocytes expressing $GABA_A$ receptor. The one possibility is that Rc might open endogenous Cl^- channels in *Xenopus* oocytes, and that the extra inward current exhibited by ginsenoside treatment in the presence of GABA might be derived from the endogenous Cl^- current, since in a previous study we showed that several ginsenosides activate endogenous Ca^{2+}-activated Cl^- channels via phospholipase C and by intracellular Ca^{2+} mobilization (Choi *et al.*, 2001). However, this may be not the case because the $GABA_A$ receptor channel blocker picrotoxin abolished Rc-induced I_{GABA} enhancement. Otherwise, the extra inward currents elicited by ginsenoside Rc treatment in the presence of picrotoxin were unaffected (Fig. 5A).

Another possibility is that Rc might act on GABA-binding sites in the GABA receptor in bicuculline sensitive manner and might facilitate the delivery of GABA to its binding site by stimulating GABA movement into $GABA_A$ receptor on the plasma membrane. Similarly, a report by Kim *et al.* (2001) showed that Rc increases the affinity of specific $[^3H]$ muscimol binding in the membrane fraction of the rat cortex following chronic infusion. However, to elucidate the exact mechanism underlying Rc-induced I_{GABA} increase, further experiments are required to determine the location of the ginsenoside interaction site in the $GABA_A$ receptor.

In summary, this study shows that ginsenosides enhance I_{GABA} in oocytes expressing human $GABA_A$ receptor. These results indicate that ginsenosides may regulate the $GABA_A$ receptor and hint that this regulation may be associated with the pharmacological actions of *Panax* ginseng.

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