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Effects of Majonoside-R2 on Pentobarbital Sleep and Gastric Lesion in Psychologically Stressed Mice

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HUONG, N. T. T., K. MATSUMOTO, K. YAMASAKI, N. M. DUC, N. T. NHAM AND H. WATANABE. *Effects of majonoside-R2 on pentobarbital sleep and gastric lesion in psychologically stressed mice.* PHARMACOL BIOCHEM BEHAV **53**(4) 957-963, 1996. —The effects of Vietnamese ginseng (VG) and its major constituent majonoside-R2 on pentobarbital-induced sleep and gastric lesion in psychologically stressed mice were examined. Psychological stress exposure for 30 min significantly decreased the duration of pentobarbital (50 mg/kg, IP)-induced sleep in mice. VG extract (50 mg/kg, PO), VG saponin (25 mg/kg, PO), and majonoside-R2 (3.1-12.5 mg/kg, PO and IP) had no effect on pentobarbital sleep in unstressed control mice, but these drugs significantly recovered pentobarbital sleep decreased by psychological stress to the level of unstressed control animals. On the other hand, *Panax ginseng* (PG) extract (50-100 mg/kg, PO) failed to affect pentobarbital sleep in psychologically stressed mice. The effect of majonoside-R2 on psychological stress-induced decrease in the hypnotic activity of pentobarbital was significantly blocked by flumazenil (1 mg/kg, IV), a selective benzodiazepine antagonist. Diazepam (0.1 mg/kg, IP) significantly prolonged pentobarbital sleep in unstressed and psychologically stressed groups, and the effect of diazepam was significantly attenuated by the same dose of flumazenil. Naloxone (0.5-5 mg/kg, IP), an opioid antagonist, had no effect on pentobarbital sleep in unstressed or psychologically stressed animals. Psychological stress exposure for 16 h caused gastric lesion in mice. VG extract (25-50 mg/kg, PO) and majonoside-R2 (6.2-12.5 mg/kg, PO), as well as diazepam and naloxone, produced the protective action on gastric lesion in psychologically stressed mice. These results suggest that VG and its major constituent majonoside-R2 have the protective effects on the psychological stress-induced pathophysiological changes and that benzodiazepine receptors are partly implicated in the effects of majonoside-R2.

Psychological stress Vietnamese ginseng extract Vietnamese ginseng total saponin Majonoside-R2
Pentobarbital-induced sleep Gastric lesion

VIETNAMESE ginseng (VG), as well as *Panax ginseng*, has been used as a tonic and/or panacea in Vietnam (24). VG contains the same saponins as *Panax ginseng* (PG), but the major difference between the constituents of these two ginsengs is that ocotillol-type saponins such as majonoside-R2 exist in VG but not PG (23). The effects of PG on the central nervous system have been extensively investigated, and numbers of studies have demonstrated the neuromodulatory action of PG (33,35,37). Compared with PG, however, few reports are available on the pharmacological actions of VG.

Various stressful manipulations are known to cause pathophysiological changes in laboratory animals. For example,

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foot shock, forced swimming stress, and restraint stress change barbiturate-induced sleep, produce antinociception, or cause gastric lesion in rodents (3,7,39). Emotional factors such as anxiety and fear appear to play an essential role in these pathophysiological effects of stressful stimuli (9,12,13,36). Recently, we found that VG saponin and majonoside-R2 attenuated psychological stress- and foot shock stress-induced antinociception in mice (25). In the present study, we investigated the effects of VG and majonoside-R2 on pentobarbital-induced sleep and gastric lesion formation in psychologically stressed mice to further clarify the antistress effect of VG.

METHODS

Animals

Male 5-week-old ddY mice (Japan SLC, Shizuoka, Japan) were used for the experiments. The animals were housed in groups of 20–25 per cage for at least 1 week before starting the experiments. Housing conditions were thermostatically maintained at 24 ± 1°C and humidity at 55 ± 5%, with a 12 L : 12 D cycle (lights on: 0730–1930 h). Food and water were given ad lib. All experiments were done in compliance with the Guide for Animal Experiments, Toyama Medical and Pharmaceutical University.

Apparatus

The communication box devised by Ogawa et al. (27) was used to expose mice to psychological stress (Fig. 1). It consists of 25 compartments (10 × 10 × 40 cm each) with transparent Plexiglas walls and stainless steel grid floor (0.5 cm diameter rods 1 cm apart from each other). Mice were individually placed in the A and B compartments. The floor grids of the B compartments were covered with Plexiglas plates. Intermittent electric shocks (1 mA, 1 s duration, 4 s intershock interval for 30 min in pentobarbital hypnosis experiments and 1.6 mA, 10 s duration, 110 s intershock interval for 16 h in gastric lesion experiments) were delivered through the grids by a shock generator (Muromachi-Kikai Co., Ltd., Tokyo, Japan) according to the method described by Nomura et al. (26). Thus, only the mice (sender) in the A compartments received foot shock through the grid floor but the animals in the B compartments (responder) were exposed to psychological stress by watching and hearing the struggle, jumping, and vocalization of sender mice in the adjacent compartments.

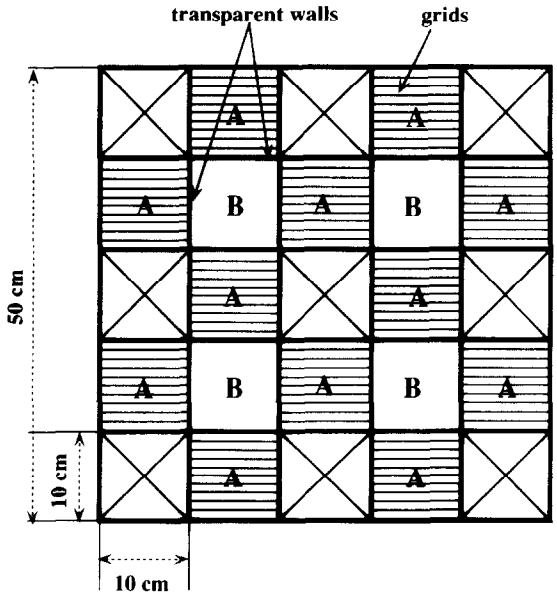

FIG. 1. Schematic drawing of the communication box used to expose mice to psychological stress. Mice were placed individually in each compartment (A and B). The animals in the A compartments received foot shock through the grid floor and those in the B compartments were exposed to psychological stress by watching the behavior or hearing the vocalization of the animals in the A compartments.

Preparation of Vietnamese Ginseng Crude Extract, Total Saponin Fraction, Majonoside-R2, and Panax Ginseng *Crude Extract*

Powdered Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv. Araliaceae) root and rhizome (5 years old) were extracted 4 times with 96%, 48%, 24%, and 0% v/v ethanol, respectively, using a percolation method. The combined extracts were evaporated under reduced pressure and then lyophilized to yield Vietnamese ginseng crude extract (VG extract; yield: 41.2%). Following extraction with ethyl ether to remove lipids from the extract, water-saturated *n*-butanol was added. The *n*-butanol extract was then evaporated to dryness to yield the total saponin fraction (VG saponin; yield: 13.2%). Majonoside-R2 was purified from the fraction as described previously (yield: 5.29%) (23). *Panax ginseng* crude extract (PG extract) was prepared from powdered PG root (Tochimoto Pharm. Co., Ltd., Osaka, Japan) in the same way as described above (yield: 35.7%).

Pentobarbital-Induced Sleep

Immediately after exposing mice to psychological stress for 30 min, 50 mg/kg pentobarbital sodium (Tokyo Kasei, Co., Ltd., Tokyo, Japan) was injected intraperitoneally. Sleeping time was taken as the period between the loss of the righting reflex and its return (22,29).

Psychological Stress-Induced Gastric Lesion

The experimental procedure was the same as that described by Nomura et al. (26). Briefly, mice were fasted for 24 h and then, randomly divided into two groups (by body weight). The one was subjected to the psychological stress for 16 h and the other was placed in each compartment individually without being exposed to psychological stress or foot shock for the same period as the psychologically stressed group. The animals were killed by decapitation and their stomachs were rapidly removed and immersed in saline containing 1% formaldehyde. The gastric mucosa was exposed by cutting along the greater curvature, washed lightly with saline, and inspected macroscopically. The gastric lesion severity was scored according to the criteria reported by Tsukamoto et al. (38) (score: 0 = no pathology; 1 = mucosal edema; 2 = petechia; 3 = gross mucosal edema; 4 = severe erosion; 5 = perforated ulcer). The number of animals with gastric lesion score of more than 2 was also recorded to calculate lesion incidence.

Drug Administration

VG extract, VG saponin, majonoside-R2, and PG extract were dissolved in distilled water and administered orally (PO) 1 h before stress exposure. In some experiments, majonoside-R2 or naloxone HCl (Sigma Chem., St. Louis, MO) was dissolved in saline and injected intraperitoneally (IP) 30 min and 10 min before stress exposure, respectively. Diazepam (Cercine®, Takeda Chemical Industries Ltd., Osaka, Japan)

was dissolved in saline containing 40% propylene glycol, and administered PO 1 h before stress exposure or injected IP 30 min before stress exposure. Flumazenil (Anexate®, Roche Co. Ltd., Basel) was injected IV (into tail vein) just before stress exposure. All drugs were administered in a constant volume of 0.1 ml/10 g body weight.

Statistical Analysis

Gastric lesion incidence and lesion severity were analyzed with Fisher's Exact probability test and Kruskal-Wallis analysis of variance (ANOVA) followed by Dunn's test, respectively. The duration of pentobarbital-induced sleep was analyzed with two-way or three-way ANOVA followed by Tukey's test for multiple comparison among groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effects of VG Extract, VG Saponin, and Majonoside-R2 on Psychological Stress-Induced Decrease in Pentobarbital Sleeping Time

As summarized in Table 1, psychological stress exposure for 30 min significantly decreased the duration of pentobarbital sleep in mice. VG extract (50 mg/kg, PO), VG saponin (25 mg/kg, PO), or majonoside-R2 (3.1, 6.2, and 12.5 mg/kg, PO or IP) had no effect on the pentobarbital-induced sleep in unstressed control mice, but they significantly recovered the hypnotic activity of pentobarbital decreased by psychological stress to the level of unstressed control mice. PG extract (50 and 100 mg/kg) failed to recover the psychological stress-induced decrease in pentobarbital sleep, $F(2, 136) = 2.265$, $p = 0.108$. A two-way ANOVA revealed significant effect of diazepam treatment on pentobarbital sleep in unstressed and psychologically stressed animals, $F(3, 95) = 49.288$, $p < 0.01$, and pentobarbital sleep in 0.1 and 0.5 mg/kg diazepam-treated groups were significantly longer than that in vehicle treated group. On the other hand, significant naloxone × stress interaction was observed, $F(3, 105) = 3.281$, $p < 0.05$, but the effect of naloxone (0.5–5 mg/kg, IP) treatment was not statistically significant, $F(3, 105) = 1.044$, $p = 0.376$ (Table 2).

A two-way ANOVA revealed a significant diazepam × flumazenil interaction, $F(1, 80) = 15.114$, $p < 0.01$, and a significant majonoside-R2 × flumazenil interaction, $F(1, 83) = 7.003$, $p < 0.05$] (Fig. 2). Flumazenil (1 mg/kg, IV) by itself had no effect on pentobarbital sleep but it significantly blocked the effects of diazepam (0.1 mg/kg, IP) and majonoside-R2 (3.1 mg/kg, IP) on the pentobarbital sleep decreased by psychological stress. A three-way ANOVA revealed a significant stress exposure × majonoside-R2 × flumazenil interaction, $F(1, 83) = 6.319$, $p < 0.05$, but no significant stress exposure × diazepam × flumazenil interaction, $F(1, 80) = 0.396$, $p > 0.05$.

Effects of VG Extract and Majonoside-R2 on the Psychological Stress-Induced Gastric Lesion

Psychological stress exposure for 16 h significantly increased the gastric lesion incidence (76.9 and 17.5%, in stressed and unstressed mice, respectively) and gastric lesion severity (Table 3). Pretreatment with VG extract (25 and 50

TABLE 1

EFFECTS OF VG EXTRACT, VG SAPONIN, AND ITS MAJOR CONSTITUENT MAJONOSIDE-R2 ON PSYCHOLOGICAL STRESS-INDUCED DECREASE IN THE HYPNOTIC ACTIVITY OF PENTOBARBITAL IN MICE

Drugs	Dose (mg/kg)	Sleeping Time (min)		
		Unstressed	Stressed	Interaction Between Stress and Drug
Experiment I				
Vehicle		73.0 ± 1.9	56.2 ± 2.3*	
VG extract	25	65.4 ± 3.0	56.5 ± 3.1	$F(3, 169) = 5.696$, $p < 0.01$
	50	70.3 ± 4.5	79.5 ± 5.3‡	
	100	77.7 ± 6.3	72.0 ± 4.3	
PG extract	50	76.7 ± 3.6	65.2 ± 4.3	$F(2, 136) = 4.693$, $p < 0.05$
	100	64.3 ± 4.5	65.8 ± 3.5	
Experiment II				
Vehicle		70.2 ± 1.6	56.9 ± 1.9*	
VG saponin	12.5	68.9 ± 2.6	65.0 ± 2.8	$F(2, 118) = 4.064$, $p < 0.05$
	25	75.5 ± 4.0	71.3 ± 4.7‡	
Majonoside-R2	6.2	73.4 ± 2.0	80.0 ± 6.3‡	$F(2, 118) = 4.105$, $p < 0.05$
	12.5	76.4 ± 4.0	69.9 ± 4.0§	
Experiment III				
Vehicle		73.1 ± 2.9	58.3 ± 2.2†	
Majonoside-R2	3.1	71.6 ± 3.7	80.7 ± 4.2‡	$F(3, 101) = 5.523$, $p < 0.01$
	6.2	66.9 ± 2.8	72.2 ± 1.5§	
	12.5	74.5 ± 4.3	74.6 ± 4.1§	

Mice were divided into two groups and only the one group was exposed to psychological stress for 30 min. Test drugs were administered PO 1 h before stress exposure except for Experiment III. In Experiment III, majonoside-R2 was injected IP 30 min before stress exposure. Each datum represents the mean ± SEM of 12–15 mice. *$p < 0.01$ and †$p < 0.05$ compared with respective unstressed group. ‡$p < 0.01$ and §$p < 0.05$ compared with respective vehicle treatment (Tukey's test).

TABLE 2

EFFECTS OF DIAZEPAM AND NALOXONE ON THE DECREASE IN THE HYPNOTIC ACTIVITY OF PENTOBARBITAL BY PSYCHOLOGICAL STRESS IN MICE

Drugs	Dose (mg/kg)	Sleeping Time (min)		Interaction Between Stress and Drug
		Unstressed	Stressed	
Experiment I				
Vehicle		70.4 ± 2.3	57.4 ± 2.1*	
Diazepam	0.05	68.4 ± 3.0	68.4 ± 2.7	$F(3, 95) = 1.289, p > 0.05$
	0.10	90.9 ± 2.9†	78.6 ± 5.9†	
	0.50	141.5 ± 15.7†	118.7 ± 9.0†	
Experiment II				
Vehicle		73.5 ± 3.4	55.8 ± 2.7*	
Naloxone	0.5	67.8 ± 3.5	68.6 ± 3.0	$F(3, 105) = 3.281, p < 0.05$
	1.0	71.0 ± 2.9	69.1 ± 2.6	
	5.0	69.5 ± 3.9	66.0 ± 2.9	

Mice were divided into two groups and only the one group was exposed to psychological stress for 30 min. Diazepam and naloxone were injected IP 30 min and 10 min before stress exposure, respectively. In Experiment I, $F_{\text{diazepam treatment}}(3, 95) = 49.288, p < 0.01$, $F_{\text{stress}}(1, 95) = 8.679, p < 0.01$. In Experiment II, $F_{\text{naloxone-treatment}}(3, 105) = 1.044, p > 0.05$, $F_{\text{stress}}(1, 105) = 6.234, p < 0.05$. Each datum represents the mean ± SEM of 10–18 mice. *$p < 0.01$ compared with unstressed group. †$p < 0.01$ compared with vehicle treatment.

mg/kg) and majonoside-R2 (6.2 and 12.5 mg/kg) significantly reduced the incidence and severity of gastric lesion in psychologically stressed mice without affecting these parameters in unstressed control animals. The reference drugs diazepam (10 mg/kg, PO) and naloxone (5 mg/kg, IP) also showed the protective actions on the psychological stress-induced gastric lesion, while PG extract (50 mg/kg) exhibited no statistically significant protective effect on the gastric lesion.

DISCUSSIONS

In the present study, VG and its major component majonoside-R2 showed the protective effects on the psychological stress-induced gastric lesion, and recovered the stress-induced decrease in the hypnotic activity of pentobarbital to the normal level, whereas PG extract had no effect on these pathophysiological changes.

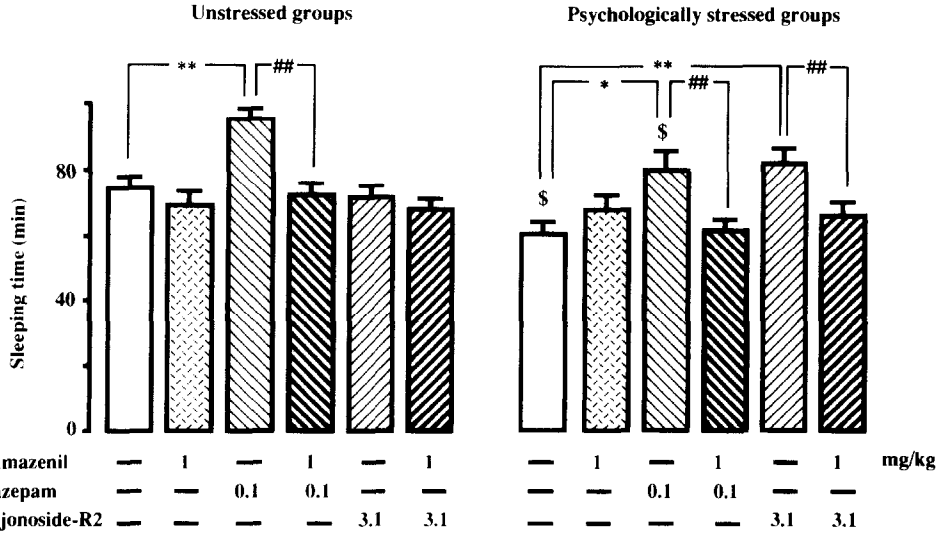

FIG. 2. Effects of diazepam and majonoside-R2 on psychological stress-induced decrease of pentobarbital sleep and antagonistic action of flumazenil on the effects of diazepam and majonoside-R2. Diazepam or majonoside-R2 was administered IP 30 min before stress exposure. Flumazenil was injected IV just before stress exposure. The unstressed animals were treated with test drugs in the same way as the stressed mice. Each value is the mean ± SEM of 12 mice. *$p < 0.05$ and **$p < 0.01$ compared with the respective vehicle control groups, ##$p < 0.01$ compared with the corresponding nonflumazenil groups and \$$p < 0.05$ compared with the corresponding unstressed groups (Tukey's test).

TABLE 3

EFFECTS OF VG EXTRACT, VG SAPONIN, AND MAJONOSIDE-R2 ON PSYCHOLOGICAL STRESS-INDUCED GASTRIC LESION

Drugs	Doses (mg/kg)	Lesion Incidence		Lesion Severity	
		Unstressed	Stressed	Unstressed	Stressed
Vehicle		7/40	30/39*	1.4 ± 0.2	3.5 ± 0.3†
VG extract	25	1/12	1/12‡	1.1 ± 0.4	1.3 ± 0.3‡
	50	1/14	3/14‡	1.1 ± 0.4	1.8 ± 0.4§
Majonoside-R2	6.2	3/14	1/13‡	2.1 ± 0.4	0.9 ± 0.3‡
	12.5	1/12	2/12‡	1.1 ± 0.4	1.5 ± 0.4‡
PG extract	50	1/14	7/14*	1.1 ± 0.3	2.3 ± 0.5
Naloxone	5	3/14	2/14‡	1.8 ± 0.6	2.0 ± 0.4§
Diazepam	10	3/14	4/12‡	1.9 ± 0.4	1.8 ± 0.5§

Mice were divided into two groups and only the one group was exposed to psychological stress for 16 h as described in the text. Gastric lesion incidence was expressed as the ratio of the number of animals with lesion score of >2 to the number of animals used. Lesion severity was expressed as the mean score ± SEM of 12–14 mice. Test drugs except naloxone were administered PO 1 h before stress exposure. Naloxone was injected IP 10 min before stress exposure. ‡$p < 0.01$ and §$p < 0.05$ compared with the vehicle treatment. *$p < 0.05$ and †$p < 0.01$ compared with the corresponding unstressed group. Lesion incidence and lesion severity were analyzed with Fisher Exact probability test and Kruskal-Wallis test followed by Dunn's test, respectively.

Although Vietnamese ginseng contains the same ginsenosides (ginsenoside-Rb1, -Rg1, -Rd, and -Re) as *Panax ginseng*, new saponin compounds and ocotillol-type saponins, especially majonoside-R2, have been isolated from the Vietnamese ginseng saponin fraction but not from *Panax ginseng* saponin fraction (23). Thus, these differences between the chemical compositions of Vietnamese ginseng and *Panax ginseng* may explain the action profiles of Vietnamese ginseng differing from those of *Panax ginseng*. The present finding that majonoside-R2, as well as Vietnamese ginseng saponin, attenuated the pathophysiological effects of psychological stress supports this idea, and gives further evidence that majonoside-R2 plays an important role in the effects of Vietnamese ginseng (25).

Psychological stimuli such as noise, fear, anxiety, etc., increase arousal level and cause insomnia in humans (21). In experimental animals, increasing evidence suggests that various kinds of stressful stimuli induce changes in the hypnotic activities of barbiturates (3,22,34,39). Opiatergic, dopaminergic, and GABAergic systems appear to be involved in these pathophysiological changes following stress exposure (1,4,6, 14). The previous report from this laboratory (22) demonstrated that repeated application of forced shaking stress at low temperature decreased the hypnotic action of pentobarbital in mice and that diazepam attenuated the stress-induced decrease of pentobarbital sleep through benzodiazepine receptors, suggesting that functional changes in $GABA_A$ receptor systems participate in the decrease of pentobarbital sleep by forced shaking stress. In the present study, psychological stress exposure for 30 min also significantly decreased the duration of pentobarbital sleep in mice. Although diazepam significantly prolonged pentobarbital sleep in unstressed and psychologically stress mice and the effect of diazepam was significantly attenuated by flumazenil, no significant stress exposure × diazepam × flumazenil was observed. Thus, the contribution of $GABA_A$ systems to the psychological stress-induced decrease in pentobarbital sleep may be not so large as that to the forced shaking stress-induced decrease of pentobarbital sleep (22).

It is of interest to note that the VG extract, VG saponin, and majonoside-R2 recovered the hypnotic activity of pentobarbital decreased by psychological stress to the level of unstressed control mice and that the effect of majonoside-R2 was significantly blocked by flumazenil. Exact mechanisms underlying this apparent antagonistic interaction between majonoside-R2 and flumazenil in psychologically stressed mice remain unclear, but these findings suggest that benzodiazepine receptors participate partly in the action of majonoside-R2.

Opioid systems also appear to be involved in the pathologic states induced by stressful stimuli since naloxone, an opioid receptor antagonist, modulates the stress responses of the animal (5,28). In our previous study (25), both VG saponin and majonoside-R2, as well as naloxone, suppressed the psychological stress-induced antinociception in mice in a dose-dependent manner, suggesting possible involvement of opioid receptor mechanisms in the effects of VG saponin and majonoside-R2. Taken together, functional changes in opioid systems caused by psychological stress exposure may participate in the effect of Vietnam ginseng on pentobarbital sleep in psychologically stressed mice. However, such a possibility seems to be little, if any, because naloxone produced no statistically significant action on pentobarbital sleep in unstressed or psychologically stressed mice.

The central nervous system and the brain-gut axis have been suggested to play important roles in stress-induced gastric ulceration (8,11,20,31). In this study, both naloxone and diazepam significantly reduced the gastric lesion incidence and lesion severity in mice exposed to psychological stress for 16 h. Although controversial data have been reported regarding the roles of central opioid systems in the stress gastric ulceration (7,18), the present results suggest possible involvement of opioid and $GABA_A$ systems in the psychological stress-induced gastric lesion in mice. As well as these reference drugs, VG extract, VG saponin, and majonoside-R2 showed protective effects on gastric lesion caused by psychological stress. Taking into account the possible roles of $GABA_A$ systems in mediating the effects of VG and majonoside-R2 on pentobarbital sleep in psychologically stressed mice, it is quite interesting to speculate that $GABA_A$ systems are partially im-

plicated in the protective effect of majonoside-R2 on the psychological stress-induced gastric lesion. To clarify the exact mechanisms underlying the protective effects of majonoside-R2 on the pathophysiological changes caused by psychological stress will require further investigations.

In conclusion, Vietnam ginseng produces protective effects on pathophysiological changes following the psychological stress exposure in mice. Majonoside-R2, one of the major saponin constituents of Vietnamese ginseng, may play an important role in the effect of Vietnamese ginseng.

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