Charaterization of Ginsenosides-induced Antinociception in Mice

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ABSTRACT

We have investigated the antinociceptive efficacy of ginseng saponins in mice using 1% formalin, which induce two phases of pain (acute and tonic pains) and is known to induce a clinically related pain. Ginseng total saponins (GTS) relieved both phases of pain with ED_{50} of 162 mg/kg for acute and 92 mg/kg for tonic pain, respectively. Both protopanaxadiol (PD) and protopanaxatriol (PT) saponins did not attenuated acute phase of pain but relieved tonic phase of pain with ED_{50} of 45 mg/kg for PD saponins and 105 mg/kg for PT saponins, respectively. Moreover, ginsenoside Rc, Rd, and Re among representative ginsenosides such as Rb_1, Rc, Rd, Re and Rg_1 relieved slightly but significantly acute phase of pain and strongly attenuated tonic phase of pain but Rf relieved only tonic phase of pain. However, PD and PT saponins, and the individual ginsenosides tested except GTS did not greatly attenuate thermal noxious pain (tail-flick test). These results suggest that single ginsenoside or mixture of various ginsenosides mainly induce differential antinociception in mice.

Introduction

Ginseng is the root of *Panax ginseng* C. A. Mayer (Araliaceae), a well-known oriental folk medicine from long time ago and is used by far east, south east countries, Europe and even Russia. In north america, ginseng is also recently cultivated and is now in markets for keeping one healthy or naturopathic treatment. Ginseng is now one of the prototypical herbal medicines consumed in all around world.

Ginseng saponins or ginsenosides isolated from ginseng are main pharmacoactive molecules of ginseng (1). Ginseng saponins show a variety of efficacies such as anticancer, antihypertension, antidiabetes, antistress, antinociception, facilitating learning, and improving the weak body conditions as tonics (2). Although ginseng or ginseng saponins are thus used for multiple purposes, it is not proved exactly for its therapeutical efficacy. The cellular or molecular mechanism of ginseng action is not even known.

We demonstrated recently that ginseng root extract or ginsenoside Rf inhibits N-type (and other high-threshold) Ca^{2+} channels in rat sensory neurons with dose-dependent manner (3, 4). The

inhibitory effect of ginseng extract or ginsenoside Rf on Ca^{2+} channel activity is mediated via a pertussis toxin-sensitive GTP-binding protein (s). Interestingly, we observed that a maximal dose of ginsenoside Rf inhibits Ca^{2+} channels current in sensory neurons to the same extent (> 20 %) as maximal activation of the μ-opioid receptor by its selective agonist, DAMGO. The inhibition of Ca^{2+}-evoked neurotransmitter release from sensory neurons is known to be a key element in opioid pain inhibition in the spinal cord, and the ability of ginsenoside Rf to block N-type Ca^{2+} channels to the same extent as opioid is strongly predictive of an antinociceptive action of this ginsenoside. In particular, these results support the previous reports that ginseng saponins has antinociceptive action. However, previous reports performed analgesic experiments using only total ginseng saponins and even more did not characterize the analgesic effects of ginseng saponins.

The purpose of this study is to investigate the influence of ginseng total saponins (GTS), protopanaxadiol (PD), protopanaxatriol (PT) saponins and representative individual ginsenosides on the antinociception against pains induced by various ways such as chemicals or thermal stimulations.

Materials and Methods

Animals : ICR mouse (20-25 g) was used for analgesic experiments. 8-10 mice were used for each point of data.

Chemicals : Ginseng total saponins (GTS), PD and PT saponins, or individual ginsenosides isolated from Red ginseng root were provided from Korea Ginseng and Tobacco Research Institute. Ginseng saponins and individual ginsenosides are dissolved in saline or 1% carboxymethylcellulose (CMC).

Formalin test : A slightly modified version of the technique of Hunskaar and his colleagues was used with mice (5) [originally described by Dubuisson and Dennis (6) in rats and cats]. 1% formalin was prepared from the aqueous solution of 37 % w/w formaldehyde. In this assay, mice were introduced to the testing environment, i.e., 30㎝ high, 20㎝ diameter plexiglass box for 60 min before any injection. A mirror was placed behind the cylinders for easy observation of whole body of testing animal. They were then weighed and returned to the cylinders. After twenty minutes, i.p. injection of the test substance, 40 μl of 1 % formalin was injected just under the skin of the plantar surface of the left hind paw by use of a microsyringe with a 29-gauge needle. Mice were returned to the cylinders and immediately observed for bitings and lickings of the affected hind paw. The total time that spent bitings and lickings the left hind paw over the next 40 min was measured with a stopwatch and recorded to the nearest second in 5 min blocks during both phases as an indicator of nociception. Based on pilot data and in keeping with the literature, the first phase was defined as 0 to 10 min post-injection of formalin and the second phase as 11 min to 40 min post-injection.

Tail-flick test : The tail-flick assay was performed according to the method of Smith and

D'Amour (7) using mice. The intensity of the heat was set to give a baseline reaction latency time of 3-5 s. An automatic cut-off time of 15 s was used to prevent tissue damage. For the measurement of the basal latency time of the tail-flick response, mice were gently held with the tail positioned in the apparatus (IITC Life Science, USA, Model 33 tail-flick analgesy meter or Ugo Basile) for radiant heat stimulation. Mice were treated with test substance by i.p. Then, the tail-flick response after treated of ginseng saponins was measured for 0, 30, 60, 90, 120, and 240 min or 0 to 60 min.

Antinociception was expressed as percent antinociception calculated as follows; % antinociception = (mean of time spent bitings & lickings by control group - mean of time spent bitings & lickings by drug-treated group / mean of time spent bitings & lickings by control group) x 100. These values were then used to generate dose-response curves (DRCs). The DRCs were analyzed for slope and interpolated to ED_{50} by linear regression of probit-transformed percent analgesia scores by the method of Lichfield and Wilcoxon (1949)(8). Data were analyzed by analysis of variance (ANOVA) and Dunnetts procedure for multiple comparisons with single vehicle group was used to analyze the overall patterns of results. The level of significance was set to 5% ($p < 0.05$). Results are given as mean $\pm$ S.E.M.

Results

We did study the analgesic activity of ginseng total saponins (GTS) prepared from Korea Red Ginseng using formalin test. As shown in figure 1, the administration of 1% formalin into plantar surface of hind paw induced typical two phases of pain behavior. The first phase of pain or acute pain appears on 0-10 min after formalin injection and the second phase of pain or tonic pain appears 11-40 min following short term period of quiescent interval. Pretreatment of GTS at dose of less than 100 mg/kg in the first phase of pain and less than 50 mg/kg in the second phase of pain did not attenuate pain induced by formalin. ED_{50} was 162 mg/kg for the acute phase of pain and was 92 mg/kg for the second phase of pain.

Furthermore, we tested the antinociceptive effect of PD and PT saponins. In this experiment, less than 200 mg/kg of PD or PT saponins failed to show any inhibition of first phase of pain. The administration of only 200 mg/kg before formalin only slightly diminished the pain behavior (data not shown) such as biting or licking of hind paw during first phase of pain, respectively. However, the same amount of PD and PT saponins clearly reduced pain behavior during second phase of pain as shown in figure 2. Interestingly, PD saponins of 100 mg/kg also inhibited the second phase pain. ED_{50} of 45 mg/kg for PD saponins and 105 mg/kg for PT saponins for tonic phase of pain, respectively (Fig. 3).

We also tested the antinociceptive effect of its individual ginsenosides such as Rb_1, Rc, Rd, Re, Rf, and Rg_1 using formalin. Pretreatment of ginsenoside Rb_1 and Rg_1 did not show significant anal-

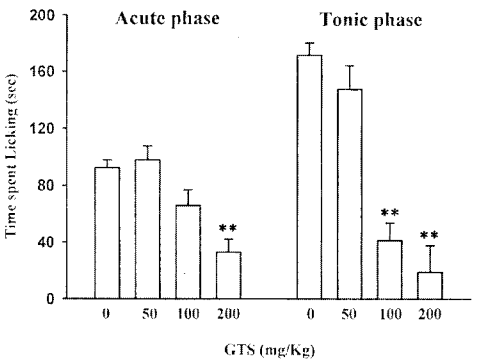

Fig. 1. The administration of 1 % formalin induces the two phases of pain pattern and pretreatment with various concentrations of GTS are usually more affecting on second phase of pain. These histograms show the acute (0-10 min) and tonic phase (11-40 min) of pain responses following the injection of formalin after pretreatment with different doses of GTS. **p < 0.01 compared to saline treated controls (by ANOVA an Dunnetts procedure for multiple comparisons).

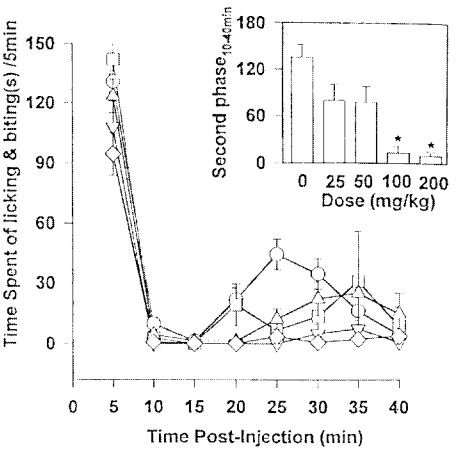

Fig. 2. The administration of 1 % formalin induces the two phases of pain pattern and pretreatment with various concentrations of PD saponins are usually affecting on second phase of pain. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of PD saponins. Pain responses were measured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1 % formalin. Pain responses are the time that spent of licking and biting(s) of the injected hind paw or leg. Each value represents the mean±SEM. *Inset;* this histograms show only the second phase during 10-40 min following the injection of formalin after pretreatment with different doses of PD saponins. *p < 0.001 compared to saline-injected controls (by ANOVA an Dunnetts procedure for multiple comparisons).

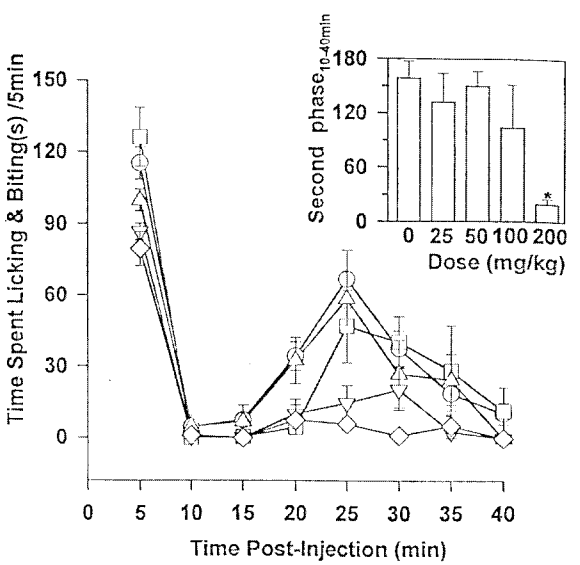

Fig. 3. The administration of 1 % formalin induces the two phases of pain pattern and pretreatment with various concentrations of PT saponins are usually affecting on second phase of pain. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of PT saponins. Pain response was measured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1 % formalin. Pain response is the time that spent of licking and biting(s) of the inhected hind paw or leg. Each value represents the mean±SEM. *Inset;* this histograms show only the second phase during 10-40 min following the injection of formalin after pretreatment with different doses of PT saponins. *p < 0.001 when compared to saline-injected controls (by ANOVA an Dunnetts procedure for multiple comparisons).

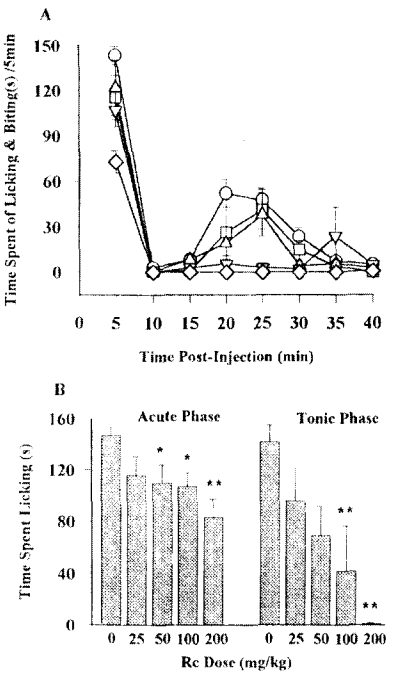

Fig. 4. The effect of ginsenoside Rc on pain induced by 1% formalin. A. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of ginsenoside Rc. Pain responses were measured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. Pain responses are the time that spent licking and biting(s) of the injected hind paw or leg. Each value represents mean ± S.E.M. B. These histograms show the acute (0-10 min) and tonic phase (11-40 min) of pain responses following the injection of formalin after pretreatment with different doses of ginsenoside Rc. *p < 0.05 or **p < 0.01 compared to 1% CMC treated controls (by ANOVA an Dunnetts procedure for multiple comparisons).

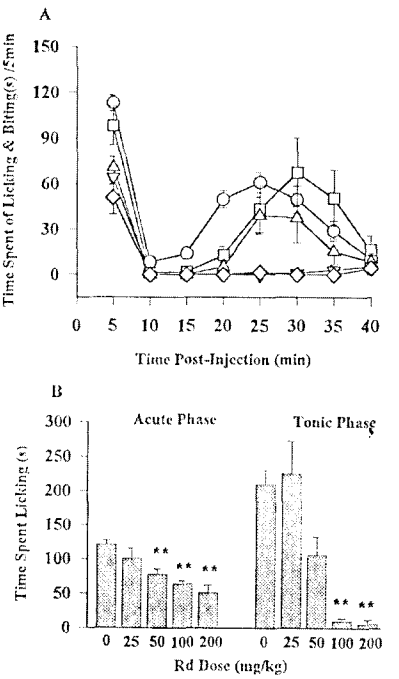

Fig. 5. The effect of ginsenoside Rd on pain induced by 1% formalin. A. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of ginsenoside Rd. Pain responses were measured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. All other details are as described in figure 2. *p < 0.05 or **p < 0.01 compared to 1% CMC treated controls (by ANOVA an Dunnetts procedure for multiple comparisons).

gesic effects in both writhing and formalin tests. However, pretreatment of each ginsenoside such as Rc, Rd, and Re at dose of 25 mg/kg in the first phase of pain and 50 mg/kg in the second phase of pain did not attenuate pain induced by formalin. These ginsenoside relieve slightly acute pain at dose over 50 mg/kg. Interestingly, pretreatment of each ginsenoside at dose of 100 mg/kg inhibited strongly the second phase of pain, indicating that these ginsenosides exert their analgesic activity by inhibiting mainly tonic pain rather than acute pain in formalin test. ED_{50} was 62 (42-90 mg/kg) for Rc, 45 (20.5-99 mg/kg) for Rd, and 82 (48-139 mg/kg) for Re in the second phase of pain, respectively (Fig. 5). Interestingly, ginsenoside Rf only attenuated the second phase of pain induced by formalin (Figs. 4-7).

We also tested whether dosages showing antinociception in formalin test relieve pain induced by

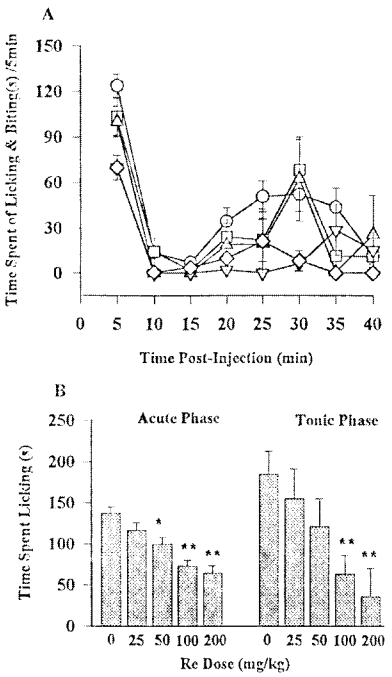

Fig. 6. The effect of ginsenoside Re on pain induced by 1% formalin. A. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of ginsenoside Re. Pain responses were measured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. All other details are as described in figure 2. *p < 0.05 or **p < 0.01 compared to 1% CMC treated controls (by ANOVA an Dunnetts procedure for multiple comparisons).

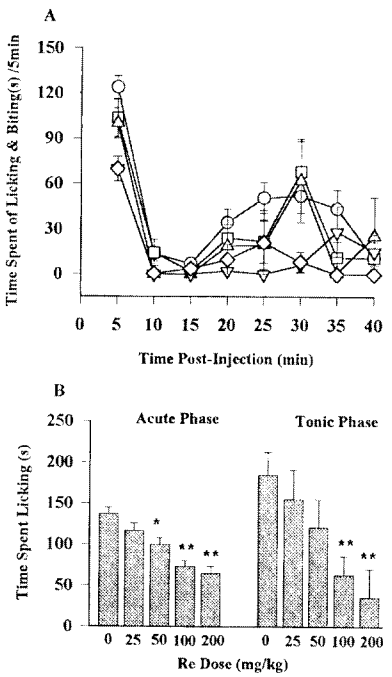

Fig. 7. The effect of ginsenoside Rf on pain induced by 1% formalin. A. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of ginsenoside Rf. Pain responses were measured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. All other details are as described in figure 2. *p < 0.05 or **p < 0.01 compared to 1% CMC treated controls (by ANOVA an Dunnetts procedure for multiple comparisons).

noxious thermal pain through tail-flick test. Interestingly, GTS showed a slight but significant analgesic efficacy at dose over 100 mg/kg (Fig. 8). However, PD or PT saponins and individual ginsenosides did not attenuate thermal pain (data not shown). We also investigated whether analgesic efficacy induced by ginsenosides is mediated via opioid receptor using opioid receptor antagonist naloxone but naloxone did not block the analgesic action of ginsenosides in writhing and formalin tests (data not shown).

Discussion

We demonstrated that pretreatment of GTS , PD and PT saponins or individual ginsenosides such as ginsenoside Rc, Rd, Re and Rf decreased the number of writhing (abdominal constriction) induced by dilute acetic acid. We also observed that the individual ginsenoside has different potency

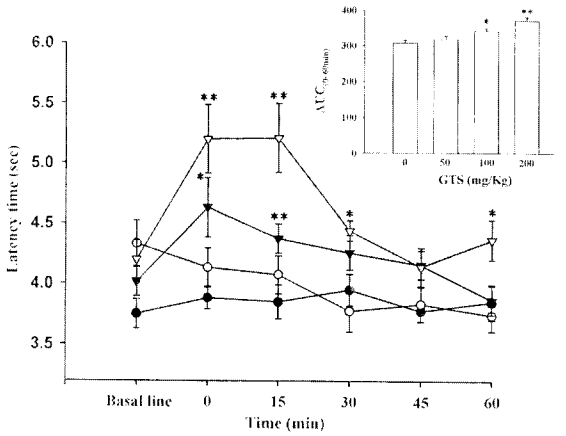

Fig. 8. The effect of GTS on noxious heat stimulus. Con (●), 50 (○), 100 (▼), or 200 (▽) mg/kg of GTS. Mice responded to a focused-heat stimulus by flicking or removing their tail. The reation time was recorded for control and for pretreated with GTS. This figure also shows time course of antinociceptive effects of pretreatment with saline or GTS on tail-flick latency. Tail-flick latency time was measured after basal latency time, 0, 15, 30, 45, and 60 min following pretreatment with GTS. *Inset;* The analgesic response was transformed into tail-flick latency time (or response) into area under curve (AUC) ± SEM. *p<0.05, **p<0.01 (by ANOVA an Dunnetts procedure for multiple comparisons).

for its inhibitory effects in writhing test. The order of analgesic potency obtained after transforming into % antinociception was Re > Rd ≥ Rc > Rf in writhing test. Ginsenoside Rb_1 an Rg_1 did not show antinociceptive activity (data not shown).

In the present study using formalin, GTS (200 mg/kg), ginsenoside Rc, Rd, or Re but not PD and PT saponins showed a significant inhibition of the first phase of pain, although in previous study ginsenoside Rf did not affect the first phase of pain with even large dose of administration (9). However, all these ginsenosides showed much higher inhibition of the second phase of pain with dose-dependent manner as shown in figures 4-7. Ginsenoside Rb_1 and Rg_1 also did not show antinociceptive activity in formalin test (data not shown). The order of analgesic potency obtained after transformation into % antinociception was Rd > Rc > Re > Rf in formalin test.

GTS relieved pain induced by noxious heat. Interestingly, PD and PT saponins and these four individual ginsenosides only showed analgesic activity in writhing and formalin tests but not in tail-flick test, suggesting that these ginsenosides induce mainly differential antinociception and these results are well consistent with previous reports (9, 10).

ED_{50} from four ginsenosides obtained through writhing test were much lower than those obtained from formalin test. This could be derived from the difference of pain intensity induced by acetic acid or formalin, since the administration of dilute formalin into under skin induces a persistent and stronger pain than that of acetic acid. These results are well consistent with previous experiment using morphine that less amounts of morphine are needed to get an analgesia in writhing test than in formalin test (11).

Treatment of ginseng total saponins induces hypothermia (12, 13). However, it is still unknown which single ginsenoside contributes the hypothermic effect. In present study we found that ginsenoside Rc and Rd were the main components for the hypothermic activity. However, treatment of ginsenoside Re in present study and treatment of ginsenoside Rf in previous study had no effect on body

temperature (9). Interestingly, ginsenoside Rc induced hypothermia for 30-60 min of treatment and induced hyperthermia after 150 min of treatment (data not shown). The explanation on body temperature fluctuation observed after treatment of ginsenoside Rc requires more investigation.

The mechanism that ginsenosides attenuate mainly chemogenic pains induced by chemicals such as acetic acid or formalin rather than thermal stimuli is not yet understood. However, recent studies showed some evidences that ginseng saponins could exert their analgesic efficacy by acting on presynaptic site(s). Nah and McCleskey (1994) reported at cellular level that ginseng saponins inhibit voltage-dependent Ca^{2+} channels in sensory neurons (3). The inhibition of voltage-dependent Ca^{2+} channels by ginseng saponins provide one possible explanation of analgesic efficacy of ginseng saponins, since sensory neurons are involved in convey of sensory information such as pain from peripheral nerve system to central nervous system. The voltage-dependent Ca^{2+} channels in sensory neurons also play an important role for the release of pain transmitters from afferent presynaptic nerve terminal into dorsal horn of spinal cord following peripheral stimulation such as formalin treatment (14), since one of contributions of opioid-induced analgesia involves the inhibition of voltage-dependent Ca^{2+} channels in sensory neurons (15). Interestingly, these regulations of voltage-dependent Ca^{2+} channels by ginseng saponins was not mediated through the inhibitory receptors such as opioid, α_2-adrenergic, GABAergic, or muscarinic receptors (3). The antinociceptive effects induced by ginsenosides were also not mediated through interaction with endogenous opioid system, since three ginsenosides were insensitive to opioid receptor antagonist naloxone (data not shown).

Furthermore, Nah *et al.*, (1995) also reported that ginsenoside Rf among several other ginsenosides such as Rb_1, Rc, Re, and Rg_1 exerts the inhibition of voltage-dependent Ca^{2+} channels in sensory neurons (4). Ginsenoside Rf inhibits voltage-dependent Ca^{2+} channels in identified nociceptive neurons and relieves pains induced by chemicals such as dilute acetic acid and formalin but not pain induced by noxious heat (9). In present study using experimental animals, both ginsenoside Rc and Re induced antinociception in writhing and formalin tests, although these two ginsenosides were relatively ineffective on voltage-dependent Ca^{2+} channels in sensory neurons (4). These results provide the possibility that ginsenosides also could use other pathway(s) that are independent of Ca^{2+} channels inhibition in sensory neurons. In fact, we recently found that ginseng saponins regulate substance P (SP)-induced nociceptive behavior. SP releases from afferent presynaptic nerve terminals into dorsal horn of the spinal cord following stimulation of peripheral nerves (14). Intrathecal (i.t.) administration of SP usually induce nociceptive behaviors in rodents as noxious stimuli were applied to their body, since SP injected i.t. acts on postsynaptic site(s) of dorsal horn of spinal cord and transfers nociceptive information to brain to induce nociceptive behaviors (16). Interestingly, co-administration of ginseng saponins with SP through i.t. route blocked the effects of SP with dose-dependent manner (17). Therefore, these results suggest that ginseng saponins act on postsynaptic site(s) as well as presynaptic site(s) in spinal cord level as opioids do. However, we can not exclude

the possibility that the analgesic efficacy of ginseng saponins also could be achieved through other pathway(s). In summary, we found that pretreatment of ginsenoside Rc, Rd, or Re through systemic administration relieves mainly chemogenic pain but spares noxious thermal pain.

Acknowledgement

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