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# Analgesic effect and related amino acids regulation of ginsenoside Rg3 in mouse pain models

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ABSTRACT

*Aims:* The present study aims to evaluate the analgesic effect of ginsenoside Rg3 in different mouse pain models.
*Main methods:* Formalin-, carrageenan- and S180 tumor cells induced mouse pain models were built in the study. The licking and biting time and PEG2 contents in the inflammatory sites were measured. The excitatory and inhibitory amino acids in the brains were determined by pre-column derivation FLD-HPLC method.
*Key finding:* We have found that ginsenoside Rg3 treated the pain phases and decreased the PGE2 in formalin and carrageenan induced models, respectively. It significantly increased the contents of EAAs (Asp and Glu) in the brains of S180 tumor inducing pain mice, meanwhile, the IAAs (Gly, Tau and GABA) decreased.
*Significance:* Our results revealed that ginsenoside Rg3 acted central and peripheral analgesic effect and regulated the inflammatory factors and pain-related amino acids. It could re-balance the abnormal EAAs/IAAs value when the pain occurred. The analgesic mechanism and the clinical application of ginsenoside Rg3 need be evaluated furtherly.

## 1. Introduction

Pain is a common symptom of many chronic and acute diseases, and one of the early signs observed by clinicians, which has been deserved more attention and proper treatment [1,2]. The drugs mostly used for the pain management are either opioids or non-steroidal anti-inflammatory drugs (NSAIDs). However, accompanied by the side effects of opioid drugs such as tolerance, addiction, excitement, drowsiness, constipation, nausea, vomiting and respiratory depression or the potential toxic effect of NSAIDs such as cardiovascular risks [3] and gastrointestinal bleedings [4], the flaws of chemical analgesics limit their further application on pain [5–7]. Thus, it is urgent to discover and develop new products for the pain treatment. Actually, the natural products have been considered as a promising remedy to support or alternate the synthetic chemicals in many clinical conditions [8,9]. More efforts have been made to introduce some herbal compounds to develop effective analgesics.

Over a long time, *Panax ginseng* C.A. is used as an important tonic in far-east Asian traditional medicine. Ginsenosides have been regarded as the active components responsible for the pharmacological and biological effects. Most ginsenosides are triterpene saponins composing of a dammarane skeleton (17 carbons in a four-ring structure) with various sugar moieties (Fig. 1) [10]. Analgesic effects of ginseng extracts have been concerned in various behavioral assays [11–13]. Ginsenoside Rc, Rd and Re could produce different antinociception in dose-dependent manner in mouse pain models. They acted the first and the second phase of pain in the formalin test and ginsenoside Re showed more potent activity in the writhing test. The ginsenosides inhibited mainly chemogenic pain rather than thermal pain by the nonopioid system in mice [14]. In addition, ginsenoside Rd had anti-inflammatory effect and mechanical pain inhibition which were due to the down-regulation of NF-κB and the consequent expressional suppressions of iNOS and COX-2 [15]. Ginsenoside Rf acted an antinociceptive effect in a rat incisional pain model by reducing the production of proinflammatory cytokines [16]. We have focused on ginsenoside Rg3, another steroid glycoside with a high molecular weight (748 Da). It is reported previously that ginsenoside Rg3 held a therapeutic effect in an incisional pain rat model. The mechanism of the analgesic effect of ginsenoside Rg3 was partly related to α2-adrenergic receptor [17].

Excitatory and inhibitory amino acids, as neurotransmitters, play important transmission and modulation roles in the central nervous system (CNS) [18–20]. Excitatory amino acids (EAAs), including aspartate (Asp) and glutamate (Glu), bind to the corresponding receptors in postsynaptic membrane to produce excitatory postsynaptic potential (EPSP), thus causing postsynaptic depolarization, so transmitting pain [21]. Levels of EAAs in the spinal cord and brain are significantly

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higher in pain models than normal animals [22,23]. Inhibitory amino acids (IAAs) including glycine (Gly), taurine (Tau) and γ-aminobutyric acid (GABA) work as the counterpart of EAAs which produce a hyperpolarisation effect due to inhibitory postsynaptic potential (IPSP). The high expression of IAAs means the decreasing of neuronal excitability [20,24]. The disturbed balance between EAAs and IAAs leads to the abnormal changes in the nociceptive transmission and modulation (central sensitization), which in turn contributes to persistence or chronicity of pain.

In the present study, we developed different mouse pain models which were used to evaluate the potential analgesic effect of ginsenoside Rg3. The formalin-induced model was used to study the different phase actions. The edema and PGE2 were detected in carrageen-induced model to know the anti-inflammatory pain. Furthermore, S180 tumor induced neuralgia model was used to determine the central analgesia effect of ginsenoside Rg3. A HPLC-FLD method was established to determine the amino acid neurotransmitters (Asp, Glu, Gly, Tau and GABA). The effect was evaluated according to the EAAs and IAAs expression after ginsenoside Rg3 was administrated. The purpose of the study was to explore the analgesic mechanism and exert the analgesic action of ginsenoside Rg3.

## 2. Materials and methods

### 2.1. Material

Ginsenoside Rg3 (purity > 93.58%) was provided by Fusheng Natural Medicine Development Co., Ltd. (Dalian, China). Asp, Glu, Gly, GABA, Tau, Hse and β-mercaptoethanol (purity > 99.0%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA,). O-Phthaldialdehyde, carrageenan and formalin was obtained from Macklin Biochemical Co., Ltd. (Shanghai, China). Methanol and acetonitrile (HPLC grade) were from Thermo Fisher Scientific (Massachusetts, USA).

### 2.2. Animal

Kunming mice (18–22 g, 4–6 weeks old) were obtained from Experimental Animal Center of Jinzhou Medical University. The animals were kept under standard environmental temperature and humidity-controlled conditions with food and water ad libitum. All procedures were conducted in accordance with National Institutes of Health Guide and International Association for the Study of Pain for laboratory animals. The protocol was approved by the Institute Animal Care and Use Committee of Jinzhou Medical University. The mice were euthanized by $CO_2$ immediately after the end of pain experiments.

### 2.3. Formalin induced pain model

To augment the role of the peripheral and systemic antinociceptive activity of ginsenoside Rg3, the mouse pain model was induced by formalin as reported previously [25]. Briefly, the mice were gently restrained and the right hind paws were injected with 20 μL of formalin solution (2.5%, in normal saline). Ginsenoside Rg3 (10, 30 and 60 mg/kg) or vehicle (0.5% CMC-Na in normal saline) were intraperitoneally (i.p.) administrated 30 min prior the test, 6 mice in each group. Immediately after formalin injection, the mice were placed in an observation chamber and pain-related behavior was quantified. The total time of licking and biting on the injected paw was monitored at 0–10 min and 10–40 min interval which were deemed as the early phase (neurogenic pain) and late phase (inflammatory pain), respectively [26].

### 2.4. Carrageenan induced pain model

Inflammatory pain induced by carrageenan is acute, nonimmune and highly reproducible [27]. Ginsenoside Rg3 and vehicle were administrated following the above dosages and route for 30 min with 6 mice each. Then, the mice received a subcutaneous injection into the plantar region of the left hind paw with 30 μL of carrageenan solution (1%, in normal saline). In the 4 h treatment, the mice were sacrificed every 1 h. The hind paws down knees were removed and weighed. The edema data are expressed as the mass difference between the injected and non-injected paws. The inflammatory paw was skinned, minced (with FSH-2A High Speed Mixer, Shanghai Zhutai, China), homogenized (in 3 mL of PBS) and centrifuged (2000 RPM, Micro 17R freezing centrifuge, Thermo scientific, USA) under 4 °C. The prostaglandin E2 (PGE2) ELISA Kit for mouse (Shanghai Mibio, China) was used for measuring the level of PGE2 in the supernatants of tissue homogenates. The double antibody sandwich method was used in PEG2 detection. The analysis was performed according to the manufacturers' guidelines. The PEG2 in the samples was combined with HRP-labeled antibody, stained in TMB solution and detected at 450 nm by a Varioskan Flash Spectral Scanning Multimode Reader (Thermo scientific, USA).

### 2.5. S180 tumor cells induced pain model

#### 2.5.1. Modeling

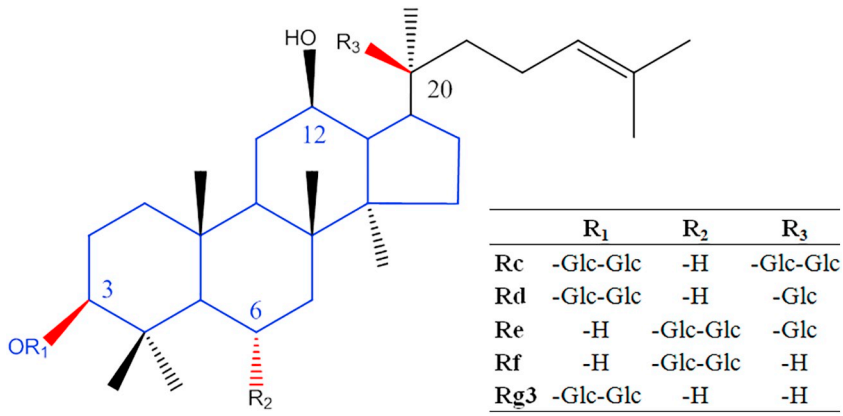
S180 tumor cells were used to establish the mice pain model as the previous paper described [28], which were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were recovered and inoculated into the abdominal cavity of a mouse. The ascites were collected and calibrated at a concentration of $1 \times 10^7$ cells/mL in fetal bovine serum (FBS, Gibco, USA). The mice were anesthetized with sodium pentobarbital (50 mg/kg, ip). The sciatic nerve on the right side was exposed at a proximal location under aseptic condition. The outer muscle layer was separated between the gluteus and the biceps femoris muscles. Immediately, 0.2 mL of the S180 cells suspension was injected intramuscularly in the obturator muscle. The operated site was irrigated with normal saline and the shaved muscle and skin were closed in layers. Following implantation assay, the tumor size was measured with a micrometer. When the tumors grew to about 100 $mm^3$, the mice showed pronounced pain symptoms like curling of the toes and cupping the paws. The mice were taken as successful pain models of nerve compression. The control mice were performed on the left side of other mice, where the sciatic nerve was exposed similarly and the same volume of FBS was injected instead of S180 cells.

#### 2.5.2. Drug treatment and sample processing

To know the regulatory effect on related amino acids (EAAs and IAAs), 60 mg/kg ginsenoside Rg3 were i.p. administrated to the S180 tumor cells induced pain models. Meanwhile, the control group and vehicle group were set, 18 cases in each group. In 4 h treatment course, 3 mice of each group were executed at 0.5 h or every 1 h intervals. The brains were removed, washed and homogenated in ice-cold normal saline. After 15 min centrifugation (3000 RPM) at 4 °C, 15 μL of Hse (1 mmol/L) and 1 mL of perchloric acid (0.4 mol/L) were added to 0.5 mL of the supernatant. Afterwards, the suspension was centrifuged (14,000 r/min). 0.2 mL of the resulting supernatant was neutralized with $Na_2CO_3$ solution and diluted to 1 mL. The pre-column derivative regent for fluorescence detection was prepared when β-mercaptoethanol, O-phthaldialdehyde and sodium tetraborate were dissolved in methanol. The reagent was kept in dark and added β-mercaptoethanol every day to preserve the derivative activity [29]. The detected solution was mixed with 15 μL of the treated sample and 5 μL of the derivative solution for 2 min in darkness at room temperature.

#### 2.5.3. Excitatory and inhibitory amino acids determination

Hse as inner standard (IS), the EAAs (Asp, Glu) and IAAs (Gly, GABA, Tau) were measured by FLD-HPLC (L-2100 pump and L-2048 FLD, Hitachi, Japan) analysis following pre-column derivatization and




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Rc | -Glc-Glc | -H | -Glc-Glc |
| Rd | -Glc-Glc | -H | -Glc |
| Re | -H | -Glc-Glc | -Glc |
| Rf | -H | -Glc-Glc | -H |
| Rg3 | -Glc-Glc | -H | -H |

**Fig. 1.** Molecular structure of different ginsenosides. Ginsenoside Re, Rd, Re, Rf and Rg3 are triterpene saponins composing of a dammarane skeleton with 2–4 glycosyl moieties.

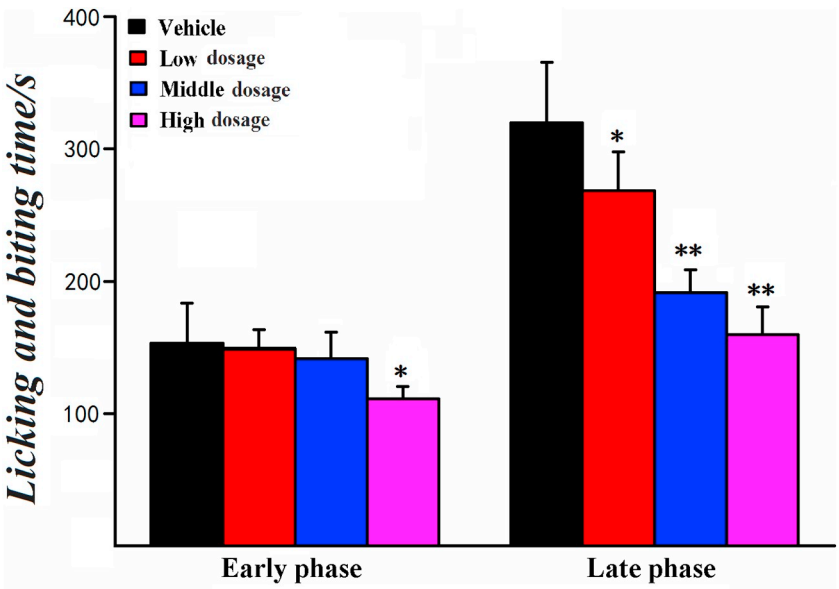



**Fig. 2.** Analgesic effects of ginsenoside Rg3 on the formalin-induced mouse pain models. 10, 30 or 60 mg/kg ginsenoside Rg3 was i.p. administrated after 30 min of formalin subcutaneous injection. 0–10 min and 10–40 min interval were deemed as the early phase and late phase, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with vehicle group (n = 6).

separation on a C18 reverse-phase chromatographic column (250 mm × 4.6 mm, 4.5 μm, at 40 °C, Cosmosil, Japan). The fluorescence was monitored at excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelength was 340 nm and 455 nm, respectively. The mobile phase was consisted of 65% of 0.05 mol/L potassium dihydrogen phosphate ($KH_2PO_4$, pH 6.0), 30% of methanol and 5% of acetonitrile. The compounds were eluted within a 30 min runtime at a 1.0 mL/min flow rate. The contents of EAAs and IAAs were calculated by chromatographic internal standard method.

### 2.6. Statistical processing

All the experiments were performed in triplicate. The results were expressed as the mean ± S.D. Differences among groups were analyzed by one-way analysis of variance (ANOVA), followed by the methods of Student's *t*-test. *p*-Value < 0.05 indicates statistically significant differences.

## 3. Results

### 3.1. Effects of ginsenoside Rg3 in formalin test

The formalin test results of ginsenoside Rg3 administration were presented in Fig. 2. Ginsenoside Rg3 showed more relieving pain behaviors than the vehicle treated mice. In the mouse models, during the early phase (0–10 min), 60 mg/kg Ginsenoside Rg3 showed a significant reduction in the licking and biting time of the behavioral response to formalin, while the low and middle dose of Rg3 had no significant action. In the late phase (10–40 min) of formalin-induced pain, ginsenoside Rg3 demonstrated a dose-dependent suppression relationship.

### 3.2. Effect of ginsenoside Rg3 in carrageenan test

We have measured the edema masses and PGE2 contents when ginsenoside Rg3 treated carrageenan-induced pain mice. As Fig. 3 showed that, there was scarcely any difference on the edema between control and Rg3 groups. Only 60 mg/kg of ginsenoside Rg3 had an edema-reducing effect before 2 h. After 3 h, the edema had grown to a proper mass (about 80 mg) no matter drug treated or not. However, when refer to PGE2, ginsenoside Rg3 showed obvious inhibitory effect in dosage and time dependence. After 4 h treatment, high dosage group (60 mg/kg) appeared a most significant inhibition of PEG2 (173.2 ± 40.4 pg), about 59.0% of vehicle group. The levels of PGE2 of middle and low dosage groups were 70.6% and 81.8% of vehicle group, respectively. Along with time, the edema increased and PGE2 decreased, but they did not show the exact inverse relationship.

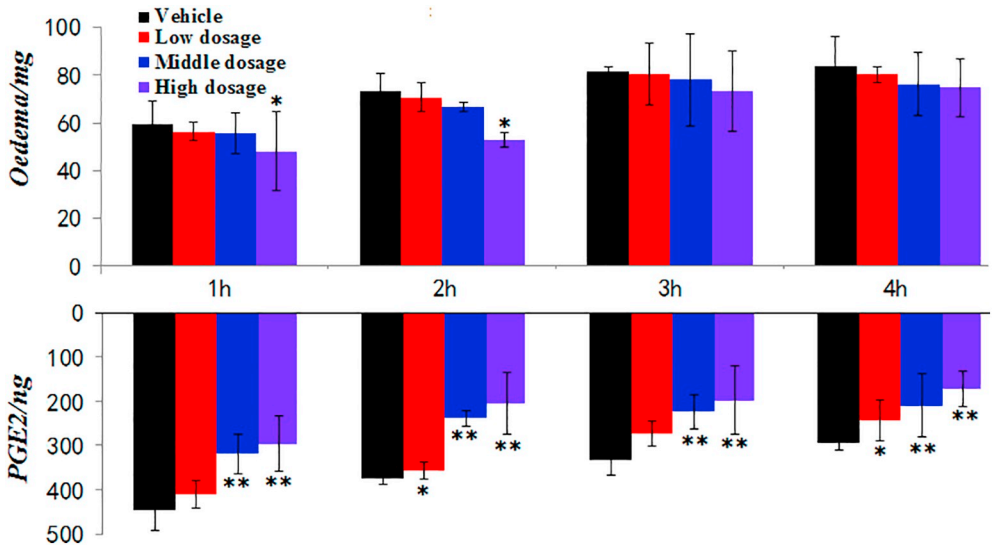



**Fig. 3.** Effects of ginsenoside Rg3 in carrageenan-induced mouse pain models. 10, 30 or 60 mg/kg ginsenoside Rg3 was i.p. administrated after 30 min of carrageenan subcutaneous injection. Edema (mg) = Mass of injected paw − Mass of non-injected paw. The PGE2 in the edema was determined by a kit according to the double antibody sandwich principle. $*p < 0.05$, $**p < 0.01$ compared to vehicle group (n = 6).

### 3.3. Effect of ginsenoside Rg3 on EAAs and IAAs

We have graphed HPLC chromatograms of the amino acids in the brain samples of S180 tumor cells-induced mouse pain models (Fig. 4), in which Asp (2.9 min), Glu (3.6 min), Hse (IS, 9.1 min,), Gly (11.0 min), Tau (18.0 min) and GABA (23.6 min) were well separated within 25 min.

The contents of amino acids were calculated by inner standard method as Table 1. Compared to the control group, the EAAs (Asp and Glu) in the brains of S180 tumor inducing pain mice were significantly increased, while the IAAs (Gly, Tau and GABA) decreased as the same time. After 4 h treatment of ginsenoside Rg3, Asp and Glu have reduced. Meanwhile, Gly, Tau and GABA increased. Among them, in the brains of pain mice, Asp, Tau and GABA had no significant difference to the control group.

Baseline value of amino acids contents in control group was set as

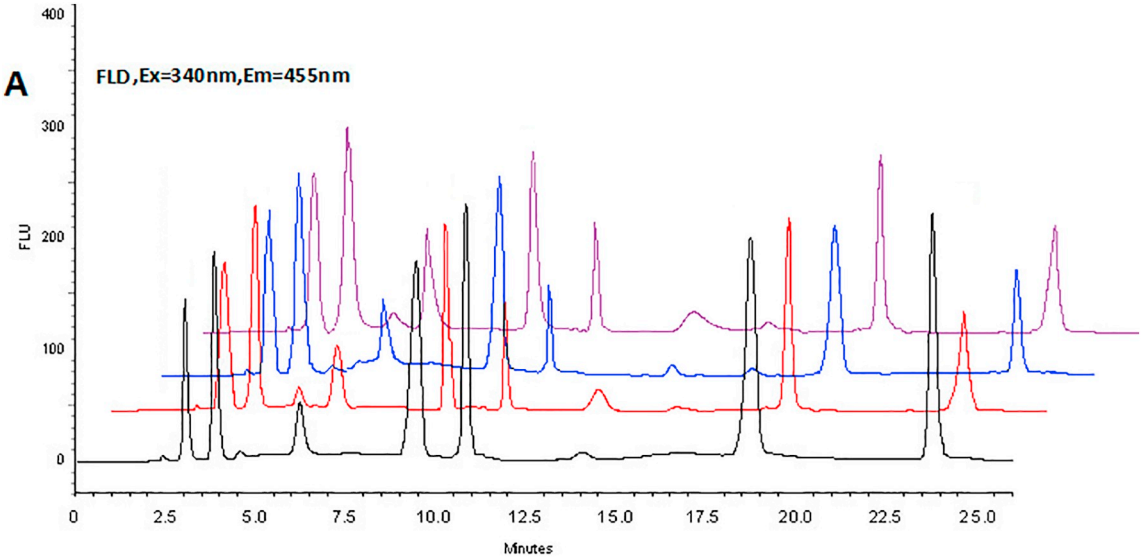



**Fig. 4.** HLPC-FLD chromatograms of different amino acids in the brain samples of S180 tumor cells-induced mouse pain models. (A) Standards (all in 1 mmol/L), (B) Control group, (C) Vehicle group, (D) Ginsenoside Rg3 group (4 h). 1 Asp, 2 Glu, 3 Gly, 4 Tau, 5 GABA, IS Hse. The amino acids were pre-column derivatizated with β-mercaptoethano and O-phthaldialdehyde. The FLU was monitored at $\lambda_{ex}$ = 340 nm and $\lambda_{em}$ = 455 nm.

**Table 1**
Contents of EAAs and IAAs in the brains of S180 tumor induced pain model mice (n = 6).

| | EAA (μmol/g) | | IAA (μmol/g) | | |
|---|---|---|---|---|---|
| | Asp | Glu | Gly | Tau | GABA |
| Control | 9.56 ± 1.04 | 12.43 ± 0.42 | 4.38 ± 0.21 | 9.27 ± 0.87 | 5.44 ± 0.64 |
| Vehicle | 13.40 ± 2.57** | 16.43 ± 0.92** | 2.83 ± 0.05** | 7.18 ± 1.29* | 4.06 ± 0.39* |
| Rg3 (4 h) | 9.36 ± 1.01 | 13.12 ± 0.63* | 3.90 ± 0.06* | 9.62 ± 1.09 | 5.68 ± 0.48 |

* $p < 0.05$ compared to control group.
** $p < 0.01$ compared to control group.

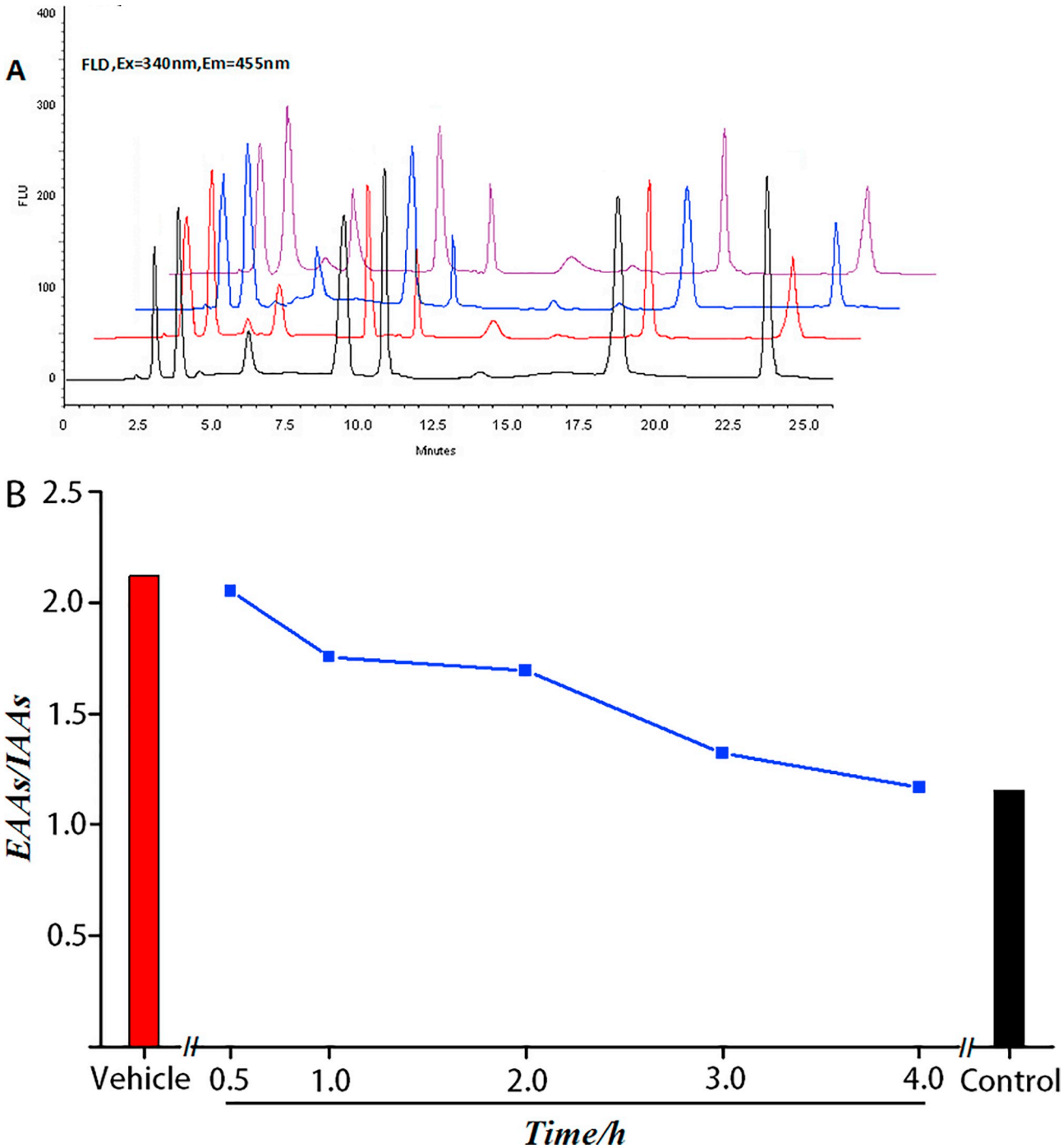



**Fig. 5.** Effects of ginsenoside Rg3 on contents of EAAs and IAAs in brains of S180 tumor cells-induced pain mice (n = 6). (A) The contents of five kinds of amino acids. Baseline level in control group was set as 100%. Data are expressed as the mean ± S.D. (B) The ratio of EAAs (Asp and Glu) an IAAs (Glu Tau and GABA).

100% [30]. The IAAs and EAAs changes were shown as Fig. 5 after ginsenoside Rg3 treated. Ginsenoside Rg3 (60 mg/kg) could significantly reduce Asp, Glu expression and increased Gly, Tau, GABA levels compared to the vehicle group. In 0.5 h, five amino acids have decreased or increased slightly. In next 0.5 h, EAAs (Asp and Glu) level decreased and two of IAAs (Gly and Tau) level increased, sharply. GABA level increased as the former 0.5 h trend. From 1 h to 4 h, the contents of amino acid returned to the baseline value gradually. Among them, GABA showed a steady fluctuation. We further measured the ratio of EAAs and IAAs, as shown in Fig. 6. In control group, EAAs/IAAs was 2.12. In ginsenoside Rg3 group, the ratio valve decreased significantly. After 4.0 h treatment, the ratio reduced to 1.17 which was in close proximity to the value of control group (1.15).

## 4. Discussion

Formalin model is widely used for evaluating the effect and mechanism of analgesic compounds in laboratory animals. The nociceptive response to formalin is biphasic. The early phase was mediated by the central effect via directly stimulating the nociceptor, and the late phase was mediated by the peripheral effect via releasing some chemical transmitters [23]. As previous reference reported, in formalin induced rat models, intrathecal ginsenosides could synergistically alleviate acute pain and facilitate the mental state [31]. We have found that

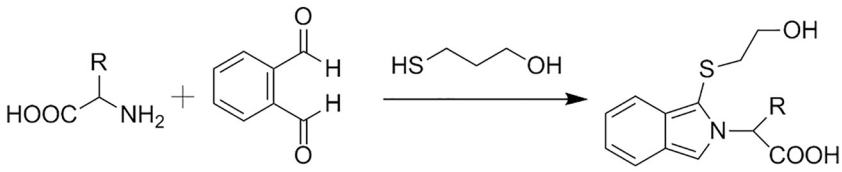



**Fig. 6.** Derivatization of amino acids in the presence of mercaptan with O-phthaldialdehyde and β-mercaptoethano.

ginsenoside Rg3 produced inhibitory effect in both phases of formalin-induced nociceptive behaviors. High dosage of ginsenoside Rg3 could reduce the pain feeling significantly. Middle and low dosages had marked analgesic effect in the late phase. In other words, ginsenoside Rg3 presented stronger peripheral analgesic effect. These results reveal that the ginsenoside Rg3 can exert analgesic effect not only on pain factors but on neurons in the brain. We further use carrageenan and S180 tumor cells induced mouse models to study the analgesic effect of ginsenoside Rg3.

Carrageenan can elicit an inflammatory pain that is characterized by a time-dependent increase in paw edema, neutrophil infiltration and increased levels of various mediators in the paw exudates [32]. Edema is one of the fundamental actions of acute inflammation [27] and PGE2 is a very important mediator of all types of inflammation [33]. The results showed that ginsenoside Rg3 didn't perform obvious edema-reduction effect but only when acted in high dosage and short time. Prostaglandins synthesized by the inducible isoform of cyclo-oxygenase sensitize peripheral nociceptors through the activation of receptors for prostaglandin E2 (PGE2) on peripheral nerve terminals [34]. In general, the acute inflammation by carrageenan injection induced a dramatic and significant increase in PGE2 production compared with the control in mouse models. Ginsenoside Rg3 showed peripheral analgesic effect by inhibition of PGE2 expression. In terms of analgesic effect, we have found that ginsenoside Rg3 exhibit a significant inhibition of inflammatory pain factors, but not induction of carrageenan-induced edema. They had no a direct relationship.

Imbalance between excitatory and inhibitory amino acids is associated with maintenance of persistent pain-related behaviors. We have monitored the changes of EAAs and IAAs associated in brain tissues using HPLC-FLD with O-phthaldialdehyde derivatization. As Fig. 6 shown, O-phthaldialdehyde and β-mercaptoethano in the presence of mercaptan reacts rapidly with primary amino acids to form intensely fluorescent derivatives [35].

S180 tumor cells induced mouse pain model has been developed by growing a malignant tumor at the sciatic nerve. In pain model, the EAAs often increase from spinal cord or cerebrospinal fluid [36]. Reduction of IAAs is associated with a central pain sensitization resulting in allodynia. Although the specific release varies a lot among each other, it reflects the inter-model differences in amino acid responses. Our data showed that the EAAs (Asp and Glu) increased and IAAs (Gly, Tau and GABA) decreased in the mouse brains of pain models. Ginsenoside Rg3 could suppress the release of Asp and Glu, which was consistent with the previous report [37]. The agent also effectively reversed the inhibition of Gly, Tau and GABA levels. It was reported that Gly and GABA released at the same synapse end, further more receptors of them were located in the same postsynaptic membrane [38]. Ginsenoside Rg3 possibly activated $GABA_A$ receptor and enhanced effects on the GABA-induced inward $Cl^-$ current at the cellular and molecular levels as well, resulting in analgesic effects. The EAAs/IAAs value appears a reliable biochemical maker of development of pain persistence and chronicity [19]. The amino acid ratio had disrupted by the arrival of ongoing activity from prolonged pathological state when S180 tumor cells-induced mouse models were developed. The abnormal changes in brain synaptic transmission and modulation (central sensitization) were in turn generating persistence or chronicity pain. Ginsenoside Rg3 could influence the release of EAAs and IAAs and maintain the EAAs/IAAs ratio at a balance level.

## 5. Conclusion

We have evaluated the analgesic effect of ginsenoside Rg3 in formalin, carrageenan or S180 tumor cells induced mouse pain models. Ginsenoside Rg3 could exhibit inhibitory effect in both early and later phase of formalin-induced models. It showed obvious inhibition of PGE2 expression in the paw edema of carrageenan-induced models. In addition, a pre-column derivatization HPLC-FLD method was established to determine EAAs (Asp and Glu) and IAAs (GLy, Tau and GABA) in the brain of S180 tumor cells-induced models. When ginsenoside Rg3 was administrated, the EAAs and IAAs were down- and up-regulated, respectively, and the EAAs/IAAs value decreased gradually. It was concluded that ginsenoside Rg3 expressed biphasic action of central and peripheral analgesic effect. It could inhibit the expression of the inflammatory pain factors and regulated the expression of amino acids. The abnormal EAAs/IAAs value was re-balanced when ginsenoside Rg3 used. We can expect a further use of ginsenoside Rg3 to relieve pain symptoms in clinical.

## Declaration of competing interest

No potential conflicts of interest were disclosed by all authors.

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