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## Research Article

## Korean Red Ginseng inhibits methamphetamine addictive behaviors by regulating dopaminergic and NMDAergic system in rodents



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## ARTICLE INFO

## Article history:

Received 7 December 2020

Received in revised form

13 May 2021

Accepted 13 May 2021

Available online 20 May 2021

## Keywords:

dopamine

drug seeking behavior

Korean Red Ginseng

methamphetamine

N-methyl-D-aspartate receptor

## ABSTRACT

**Background:** Methamphetamine (METH) is the most widely used psychostimulant and has been known to exhibit reinforcing effects even after long abstinence. We showed the inhibitory effect of Korean Red Ginseng extract (RGE) on METH-induced addictive behaviors in animal models mimicking the human drug-use pattern.

**Methods:** We first investigated the effect of RGE on the acquisition of METH-induced dependence using self-administration and conditioned place preference (CPP) tests. Additionally, further experiments such as METH-induced motivational behavior and seeking behavior were conducted. To study the underlying mechanism, dopamine receptor, dopamine transporter, and N-methyl-D-aspartate receptor were assessed through Western blot analysis.

**Results:** Treatment with RGE significantly reduced METH-induced self-administration on a fixed-ratio 1 schedule of reinforcement. It could be also decreased a progressive ratio schedule, and inhibited METH-primed reinstatement. In CPP, RGE significantly prevented the development of METH-induced CPP. Moreover, RGE not only shortened the withdrawal period clearly, but also prevented the reinstatement of CPP. RGE treatment also reversed METH-induced overexpression of dopamine transporter, dopamine receptor D1, and NMDA receptor in the nucleus accumbens.

**Conclusion:** Our findings reflect that RGE has therapeutic potential to suppress METH-induced addictive behaviors by regulating dopaminergic and NMDAergic system.

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## 1. Introduction

Addiction is a neuropsychiatric disorder caused by repeated pharmacological manipulation, and even after a long period of abstinence relapse can occur [1]. Relapse, which is a progressively incubated, cue-induced drug-seeking behavior, is a difficult problem in the treatment of drug addiction [2]. A previous clinical study showed that 61% of methamphetamine (METH) users who were discharged after METH treatment relapsed to METH use within 1 year [3]. Relapse was also observed in animal experiments. In an animal model using self-administration (SA), an amphetamine priming injection reinstated the extinguished amphetamine-

reinforced lever response in monkeys [4]. These results showed the risk of incubation of drug-seeking and revealed the need for research on the prevention of relapse after drug use has stopped.

The nucleus accumbens (NAc) was responsible for the drug-related reinforcement and drug-seeking behavior [5]. It was also the region in which the two inputs from the glutamatergic and dopaminergic neurons overlapped [6]. Specifically, glutamatergic input in the NAc has been involved in relapse after prolonged abstinence. A previous study showed that co-infusion of dopamine receptor D1 (D1DR) and N-methyl-D-aspartate receptor (NMDAR) antagonists into the NAc blocked the reinstatement (RI) induced by ventral subiculum stimulation in a d-amphetamine SA model, which was specifically implied to inhibit the plasticity of D1DR-expressing neurons [7]. Moreover, when the NMDAR1 in D1DR-expressing neurons were inactivated in mice, there was a reduction of the rewarding effect in conditioned place preference (CPP) with the loss of long-term potentiation [8]. Taken together, it is

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thought that NMDAR activity in NAc dopamine (DA) neurons following drug exposure plays a critical role in mediating drug-associated behavioral disorder.

*Panax ginseng* has been used in Asia as an herbal medicine for a long time. Previous investigations initially observed ginseng's inhibitory effects on the analgesic tolerance and physical dependence with DA receptor supersensitivity induced by morphine [9,10]. In METH-induced pharmacological changes with dopaminergic activities, pretreatment with ginseng suppressed the development of METH-induced hyperactivity, CPP, and climbing behavior while protecting against the striatal DA depletion [11,12]. These studies only evaluated the inhibitory effect on the acquisition of drug abuse; there were few studies characterizing the enhanced motivational behaviors to procure drugs and the recurring episodes of relapse to drug-seeking behaviors. Therefore, to evaluate the inhibitory effect of Korean Red Ginseng extract (RGE) on the reinforcing effect, motivational effect, craving, and relapse of addictive drugs, we conducted the METH-induced CPP test and the intravenous (i.v.) SA test using various experimental schedules in rodents. In addition, we used the standardized RGE, which exhibited an enhanced antioxidant effect and antidotal effect, to identify more potent effects on METH-induced addictive behaviors [13]. Finally, we investigated the effect of RGE on the METH-driven expression level of NMDARs as well as DA transporter and receptor in the NAc of mice exposed to a METH-primed RI of CPP.

## 2. Materials and methods

### 2.1. Animals

C57BL/6J mice (Male; 7-week-old) from Dae Han Biolink (Eumseong, Korea) were kept 10 per cage (42 × 27 × 18 cm) and male Sprague-Dawley rats (300–350 g; Orient Bio, Seoul, Korea) for the SA were housed individually. After acclimation for 1 week, rats were restricted to 90%–95% of their free-feeding body weight during food training procedure. All laboratory animals use procedures were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the institutional Animal Care and Use Committee of Sungkyunkwan University (SKKUIACUC2019-12-07-1).

### 2.2. Reagents

#### 2.2.1. Standardized red ginseng extract

The standardized RGE was provided by the Korea Ginseng Corporation (Buyeo, Chung-nam, Korea). Ginseng was steamed for 3 h (90°C–100°C), dried at 50°C to 80°C, extracted at 85°C–90°C using circulating hot water, and then filtered. The filtrate was concentrated under reduced pressure and lyophilized. Using High-performance liquid chromatography analysis, it was found that the following ginsenosides were included in RGE: 7.98 mg/g Rb1, 3.23 mg/g Rg3, 3.11 mg/g Rc, 2.89 mg/g Rb2, 2.20 mg/g Rg2s, 1.86 mg/g Re, 1.63 mg/g Rg1, 1.6 mg/g Rf, 1.17 mg/g Rh1, 1.03 mg/g Rd, and other minor ginsenosides. For the CPP experiment, the METH + RGE mice were orally administered RGE (5, 10, or 20 mg/kg). In the SA experiment, the RGE-treated rats received an intraperitoneal (i.p.) injection of 10, 20, or 40 mg/kg RGE 1 h before the METH SA sessions. Animals in the other groups received the same amount of DW or saline on the same schedule for the CPP and SA experiments, respectively.

Red ginseng extract has usually been taken orally. Thus, in order to confirm the inhibitory effect of RGE on METH CPP, which was the first experiment conducted in this study, an oral administration method known as the most common route was used. Next, in the SA test in rats, we wanted to determine whether RGE treatment

through another route could inhibit the METH-induced addictive behaviors. As a result, we used different administration routes, such as per oral for CPP and ip for SA.

#### 2.2.2. Methamphetamine

Methamphetamine hydrochloride (Sigma Aldrich, USA) was dissolved in physiological saline. It was administered i.p. injection to mice at 1 mg/kg and i.v. infusion to rats at 0.1 mg/kg/infusion.

### 2.3. Conditioned place preference

The CPP apparatus was composed of two square based Plexiglas boxes, which could be separated by a guillotine door. One box was white and had a mesh-type floor, and the other compartment was a black with a grid-type floor. The CPP test was recorded and analyzed automatically using a video-tracking system (Neuro-Vision, Pusan National University, Korea).

#### 2.3.1. Development of METH-induced conditioned place preference

To study the inhibitory effect of RGE on the acquisition of METH-induced CPP, the CPP procedures were designed as previously described [14]. The precise experimental design was shown in Fig. 1A.

#### 2.3.2. Withdrawal phase

A different set of mice was conditioned for the development of METH-induced CPP and they received RGE administered orally during WD phase. On WD days 3 and 9, preference tests were performed before the RGE treatment to measure the reduction in the METH-induced preference caused by RGE. The CPP score was also determined as described in [14]. The accurate experimental schedule was presented in Fig. 2A.

#### 2.3.3. METH-primed reinstatement

The day after last WD, the mice received RGE orally 1 h prior to a priming injection of METH and were subjected to the preference test as described for the post-conditioning test.

### 2.4. Self-administration

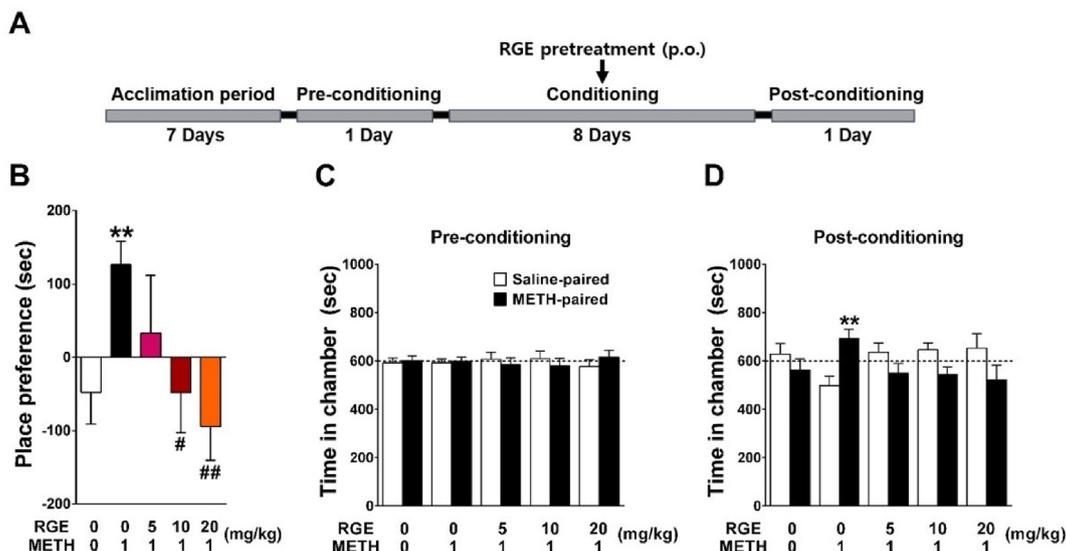
To study the inhibitory effect of RGE on the METH-induced reinforcement using a FR1 schedule (Fig. 3A), the SA test was performed as previously described with slight modification [15].

#### 2.4.1. Progressive ratio

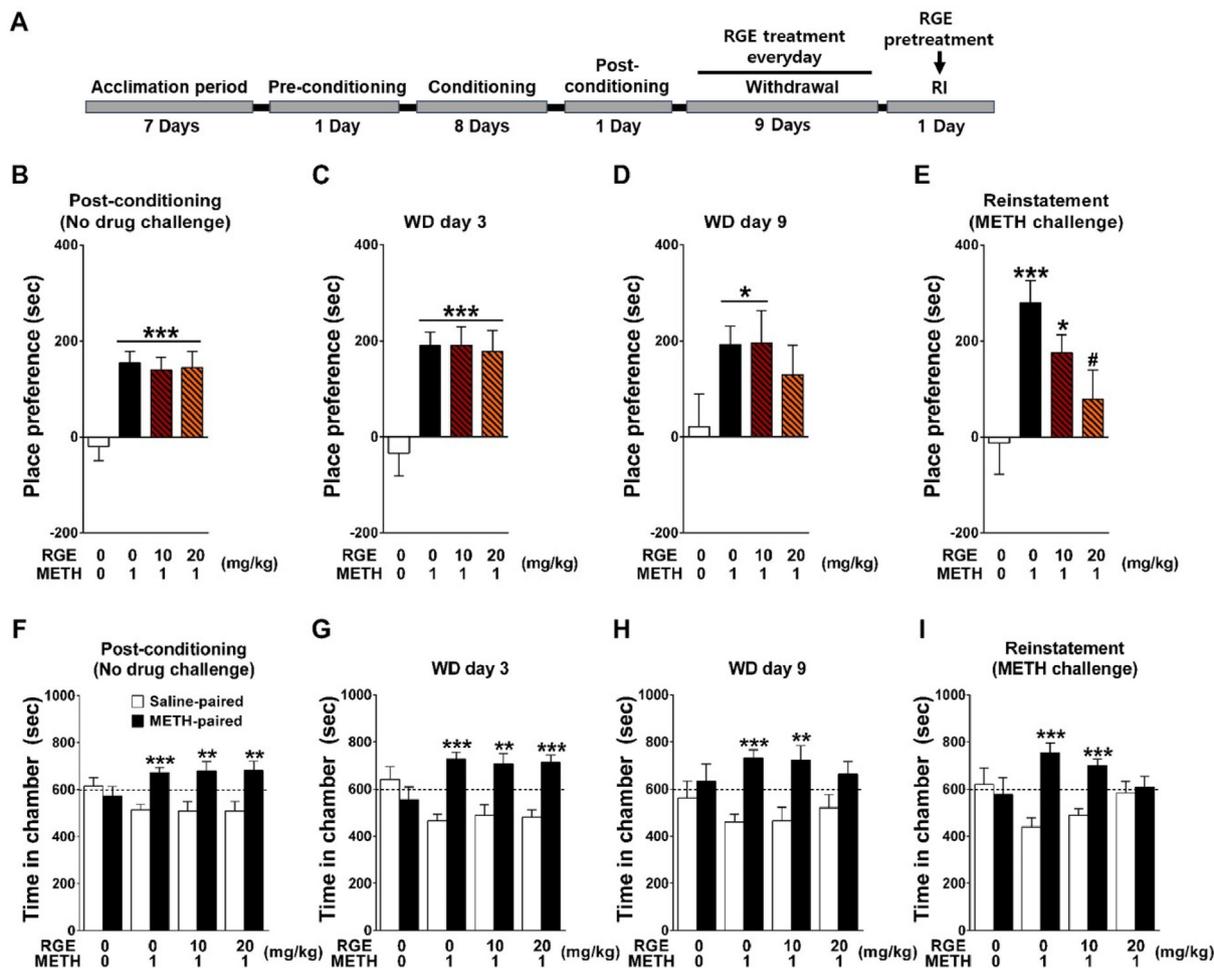
The PR schedule was used to generate the motivation to self-administer METH by increasing the response requirements [16]. Animals were given access via the PR schedule as previously described [17]. After the FR1 schedule to examine the inhibitory effect of RGE, the rats were returned to a FR1 schedule without any pretreatment. Once stable baselines were established on the FR1 schedule, the PR test session was performed for 6 h after pretreatment with RGE (Fig. 4A). If the animals did not provide the number of lever presses for the next infusion in an hour, the session was ceased.

#### 2.4.2. Extinction

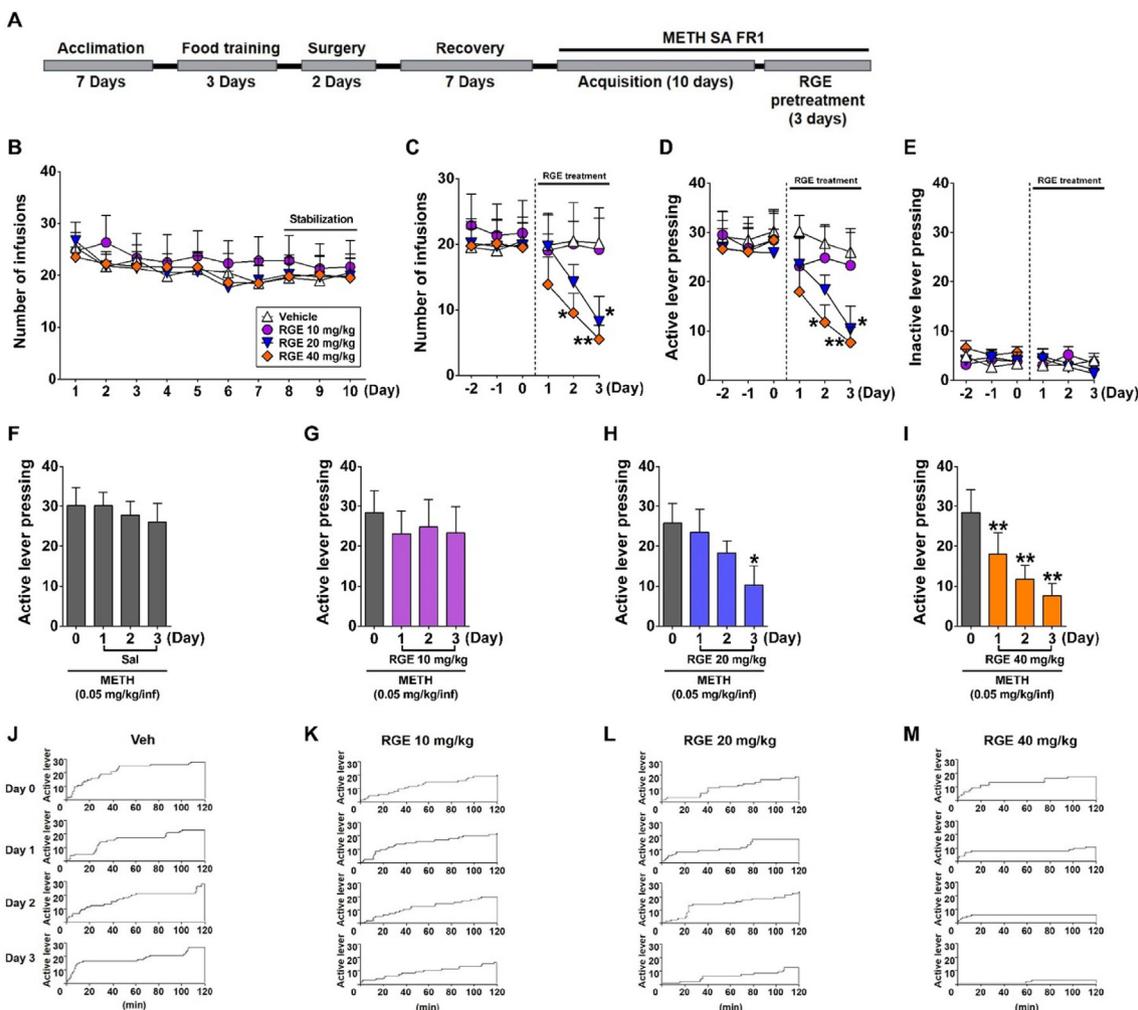
After the PR session, the rats were returned to the FR1 schedule for 1 week. Only animals that showed stable infusion patterns underwent extinction from METH access. During this period, the rats were free of reinforcement and received saline as a reward for a lever response in the operant chamber for 2 h.



**Fig. 1.** The effects of RGE on METH-induced CPP development (n = 9–16/group). (A) Experimental design. (B) Data are presented for the METH-induced CPP score. \*\*p < 0.01 compared to the control group. #p < 0.05 and ##p < 0.01 compared to the METH group. (C–D) Data are presented for the residence time in the saline-paired chamber and the METH-paired chamber on preconditioning day and post-conditioning day. \*\*p < 0.01 compared to the saline-paired chamber.



**Fig. 2.** The effects of RGE on METH-primed RI of CPP (n = 13–17/group). (A) Experimental design. (B–E) Data are presented for the CPP score on the post-conditioning day, WD days 3 and 9, and RI. \*p < 0.05 and \*\*\*p < 0.001 compared to the control group. #p < 0.05 compared to the METH group. (F–I) Data are presented for the residence time in the saline-paired chamber and the METH-paired chamber on post-conditioning day, WD day 3, WD day 9, and RI. \*\*p < 0.01 and \*\*\*p < 0.001 compared to the saline-paired chamber.



**Fig. 3.** The effects of RGE on the reinforcement of METH SA under a FR1 schedule (n = 6–8/group). (A) The experimental design. (B) Acquisition of METH SA under a FR1 schedule for 10 consecutive days. Following a stable response, rats were administered either saline or RGE intraperitoneally 1 h prior to the test session. Data are presented for (C) the number of infusions, (D) active lever pressing, and (E) inactive lever pressing. \**p* < 0.05 and \*\**p* < 0.01 compared to the vehicle group of each test session. (F–I) The number of active lever pressing for each group. \**p* < 0.05, and \*\**p* < 0.01 compared to the day 0 session of each group. (J–M) Representative individual graph of cumulative responding. Data are presented for the total number of active lever presses after pretreatment with vehicle and RGE 40 mg/kg.

**2.4.3. Reinstatement**

When the number of infusions decreased significantly to baseline, the RI test was conducted (Fig. 5A). On the test day, the animals were administered RGE 40 mg/kg 1 h before the METH priming injection, and they were immediately placed in the operant chamber. Responding was assessed on a FR1 schedule for 2 h and the lever press was recorded, but the delivery following the response did not happen. Drug-seeking behavior was defined based on the increased responding for METH during this test.

**2.5. Western blot method**

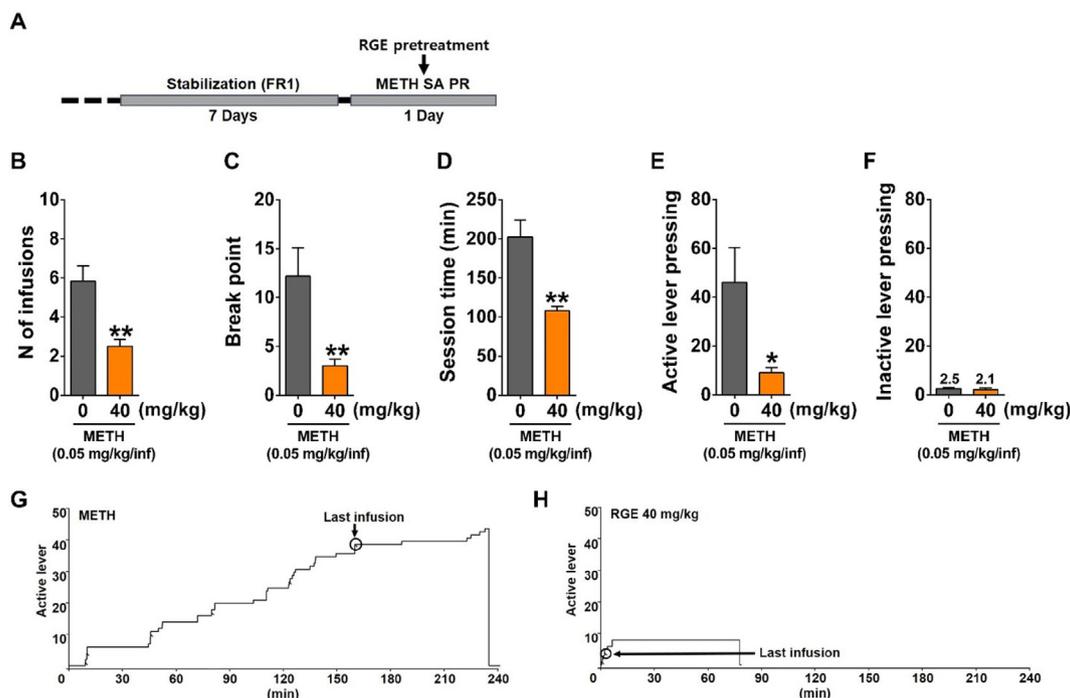
Bilateral punches of the NAc region of the mouse brain were taken and pooled after the CPP RI test. Because relapse is one of the most important and intriguing aspects seen in people with drug addiction, we investigated the underlying mechanisms to prevent METH-induced RI.

Western blot was carried out as previously reported in [18]. In this paper, the following primary antibodies were used; anti-β-actin, anti-adenylyl cyclase 1 (AC1), anti-Ca<sup>2+</sup>/calmodulin-

dependent protein kinase II (CaMKII), anti-phosphorylated CaMKII (pCaMKII), anti-dopamine transporter (DAT), anti-pDAT, anti-D1DR, anti-D2DR, anti-extracellular signal-regulated kinase (ERK), anti-pERK, anti-NR1, anti-NR2A, anti-NR2B, anti-protein kinase A (PKA), and anti-postsynaptic density protein 95 (PSD-95).

**2.6. Statistical analysis**

All data were expressed as mean ± S.E.M. The CPP score was analyzed by one-way ANOVA and the residence times in the saline-paired chamber and the METH-paired chamber were compared by an unpaired t-test. In the SA tests, the time course experiments on acquisition of METH SA were analyzed by two-way ANOVA. The number of active lever presses for each group was analyzed by one-way repeated measure ANOVA. The PR schedule and RI test were analyzed by an unpaired t-test. All behavioral data were analyzed using Fisher's least significant difference test. The Western blot data were analyzed by one-way ANOVA with Bonferroni test. Statistical significance between groups was considered at *p* < 0.05 in all statistical analysis.



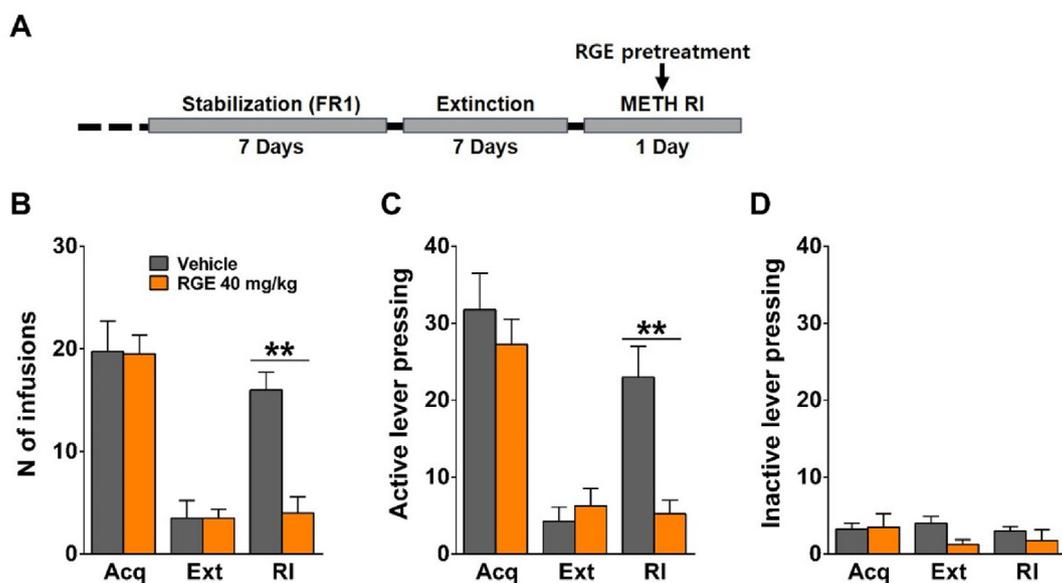
**Fig. 4.** The effects of RGE on METH SA under a PR schedule (n = 6/group). (A) Experimental design. Data are presented for (B) the number of infusions, (C) the break point, (D) the average session duration time, (E) active lever pressing, and (F) inactive lever pressing. \*p < 0.05 and \*\*p < 0.01 compared to the vehicle group. (G-H) Representative graph of cumulative responding for the effect of RGE on METH reinforcement under a PR schedule.

### 3. Results

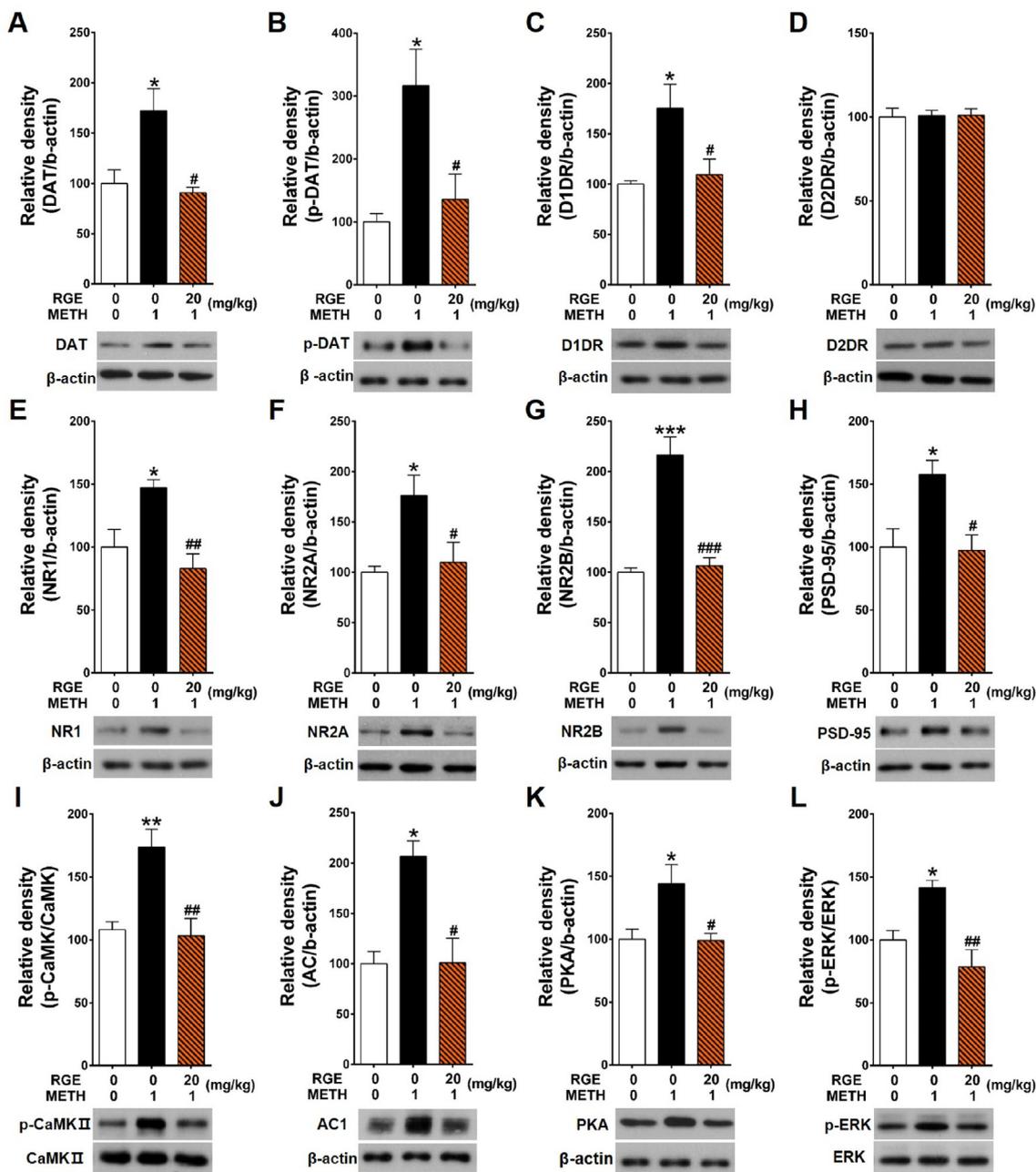
#### 3.1. Repeated RGE administration inhibited the development of METH-induced CPP

Pretreatment with RGE 5 mg/kg did not significantly decrease the METH-induced CPP score compared with that of the METH group with deionized water (DW) pretreatment. However, pretreatment with RGE 10 and 20 mg/kg showed a noticeable dose-

dependent decrease of the CPP score compared with that of the METH group with DW pretreatment (Fig. 1B, F4, 60 = 3.219, p < 0.05 and p < 0.01, respectively). Fig. 1D shows the residence time in the saline-paired chamber and the METH-paired chamber on the post-conditioning day. The METH group spent significantly more time in the METH-paired chamber (t = 3.630, p < 0.01), but RGE treatment effectively eliminated the difference in residence time between the chambers. Therefore, we examined the effects of RGE 10 mg/kg and



**Fig. 5.** The effects of RGE on METH-seeking behavior (n = 4/group). (A) The experimental design. Data are presented for (B) the number of infusions, (C) active lever pressing, and (D) inactive lever pressing. \*\*p < 0.01 compared to the vehicle group of the RI test. Acq, the last day of the acquisition session; Ext, the last day of the extinction session; RI, reinstatement.



**Fig. 6.** The effects of RGE on the expression DAT, D1DR, and NMDAR in the NAC in response to METH priming RI (n = 4–6/group). The expression levels of (A–D) Dopaminergic system-related proteins, (E–H) NMDAR subunits and PSD-95, and (I–L) enhanced activation of downstream signaling were examined by Western blot analysis. NAC punch samples were taken 1 h after METH-induced RI of CPP in mice. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001 compared to the control group. #p < 0.05, ##p < 0.01, and ###p < 0.001 compared to the METH group.

20 mg/kg, which were considered to be the most effective doses, on the withdrawal (WD) and RI sessions after METH CPP development.

### 3.2. Repeated RGE administration shortened the WD and prevented the METH-primed RI of CPP

After confirmed development of METH-induced CPP (Fig. 2B, F3, 55 = 9.468, p < 0.001), we tracked the changes in the CPP score on WD day 3 and WD day 9. The METH group and the RGE 10 mg/kg group did not show a decrease in CPP preference until WD day 9 (Fig. 2C, F3, 49 = 8.282, p < 0.001; Fig. 2D, F3, 48 = 1.964, p < 0.05). However, the RGE 20 mg/kg group showed a slight but not significant reduction in the CPP score compared to the METH group

during the WD period. On the next day, RGE 20 mg/kg significantly prevented the METH-primed RI compared to the METH group (Fig. 2E, F3, 48 = 5.406, p < 0.05). In contrast, METH and RGE 10 mg/kg produced a significant change in METH-induced CPP score compared to the control mice receiving only saline (p < 0.001 and p < 0.05, respectively). An effect was also observed on the residence times in the saline-paired chamber and the METH-paired chamber (Fig. 2F–I). When compared the residence time in METH-paired chamber, METH group on RI test had a longer time in METH-paired chamber compared to post-conditioning session. (t = 1.808, p = 0.08; not shown in data). These results suggested that if RGE was repeatedly administered even after the preference

to METH was formed, the WD period would be shortened and the METH-primed RI would also be suppressed.

### 3.3. Repeated RGE administration attenuated the acquisition of METH SA under a fixed ratio 1 schedule

Starting one day after stable responses for METH-reinforced lever pressing were attained (Fig. 3B,  $F_{27, 198} = 0.3261$ ,  $p > 0.05$ ), the rats were pretreated with 10, 20, or 40 mg/kg RGE for three days. The RGE 10 mg/kg pretreatment group did not show any significant differences compared to the METH group (Fig. 3C–E). In contrast, RGE 20 mg/kg pretreatment significantly attenuated the numbers of infusions and active lever presses on day 3 (Fig. 3C,  $F_{15, 110} = 4.614$ ,  $p < 0.05$ ; Fig. 3D,  $F_{15, 110} = 2.394$ ,  $p < 0.05$ ). Furthermore, the numbers of infusions and active lever presses were clearly decreased by RGE 40 mg/kg pretreatment from the second test session (Fig. 3C, day 2  $p < 0.05$ , day 3  $p < 0.01$ ; Fig. 3D, day 2  $p < 0.05$ , day 3  $p < 0.01$ ). However, there was no significant difference in the number of inactive lever presses among all groups (Fig. 3E,  $F_{15, 110} = 1.134$ ,  $p > 0.05$ ).

We also assessed the number of active lever presses in each group. There was no difference in the METH group and the RGE 10 mg/kg group (Fig. 3F,  $F_{1.676, 8.380} = 0.0858$ ,  $p > 0.05$ ; Fig. 3G,  $F_{1.676, 9.448} = 1.842$ ,  $p > 0.05$ ). However, the number of active lever presses was significantly decreased on the third day in the RGE 20 mg/kg group (Fig. 3H,  $F_{1.267, 6.336} = 4.860$ ,  $p < 0.05$ ). Surprisingly, there was a meaningful reduction in the number of active lever presses in the RGE 40 mg/kg group from the first day of pretreatment (Fig. 3I,  $F_{1.391, 9.738} = 15.78$ ,  $p < 0.01$ ). Fig. 3J–M shows a representative record providing a cumulative graph for the number of active lever presses at each dose. These results indicated that RGE affected the acquisition process of METH SA.

### 3.4. RGE treatment regulated the motivational effect of METH SA under a progressive ratio schedule

In the RGE 40 mg/kg group, the number of infusions, break point, session duration time, and number of active lever presses were significantly reduced compared to those in the METH group (Fig. 4B,  $t = 3.863$ ,  $p < 0.01$ ; Fig. 4C,  $t = 3.04$ ,  $p < 0.01$ ; Fig. 4D,  $t = 3.807$ ,  $p < 0.01$ ; Fig. 4E,  $t = 2.556$ ,  $p < 0.05$ ). However, there was no significant difference in the number of inactive lever presses (Fig. 4F,  $t = 0.4518$ ,  $p > 0.05$ ). Fig. 4G–H shows a representative record providing a cumulative graph for the number of active lever presses at each dose. These observations suggested that RGE treatment reduced the effectiveness of METH-induced reinforcement.

### 3.5. RGE treatment prevented the seeking behavior induced by METH-primed RI

One day after the last extinction session, we observed that pretreatment with RGE 40 mg/kg significantly reduced the numbers of infusions and active lever presses during METH-primed RI compared to those in the METH group (Fig. 5B,  $t = 5.117$ ,  $p < 0.01$ ; Fig. 5C,  $t = 4.048$ ,  $p < 0.01$ ). There was no significant difference in the number of inactive lever presses (Fig. 5D,  $t = 0.8076$ ,  $p > 0.05$ ).

### 3.6. RGE reversed the overexpression of DAT, D1DR, and NMDAR in response to METH-primed RI

The METH group showed an increase in DA transmission-related proteins such as DAT (Fig. 6A,  $F_{2, 9} = 8.539$ ,  $p < 0.05$ ), pDAT (Fig. 6B,  $F_{2, 9} = 7.864$ ,  $p < 0.05$ ), and D1DR (Fig. 6C,  $F_{2, 12} = 6.159$ ,  $p < 0.05$ ) in the NAc as compared to the control group. Moreover, the

expression of the downstream signaling molecules AC1 (Fig. 6J,  $F_{2, 6} = 11.34$ ,  $p < 0.05$ ) and PKA (Fig. 6K,  $F_{2, 12} = 6.141$ ,  $p < 0.05$ ) in the NAc was significantly increased as compared to expression in the control group. Also, the METH group showed significantly increased levels of the NMDAR subunits NR1 (Fig. 6E,  $F_{2, 9} = 8.946$ ,  $p < 0.05$ ), NR2A (Fig. 6F,  $F_{2, 15} = 6.155$ ,  $p < 0.05$ ), and NR2B (Fig. 6G,  $F_{2, 12} = 32.48$ ,  $p < 0.001$ ) and PSD-95 (Fig. 6H,  $F_{2, 9} = 7.179$ ,  $p < 0.05$ ) in the NAc as compared to the control group. As a result of overexpression of NMDARs, the intracellular phosphorylated CaMKII was increased in the METH group compared to the control group (Fig. 6I,  $F_{2, 12} = 10.87$ ,  $p < 0.01$ ). Phosphorylation of ERK was also increased in the METH group compared to the control group (Fig. 6L,  $F_{2, 9} = 11.32$ ,  $p < 0.05$ ). In RGE 20 mg/kg group, on the contrary, the METH-induced increases of all proteins except D2DR were significantly reduced ( $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ ). D2DR did not represent any changes between all groups (Fig. 6D,  $F_{2, 12} = 0.01$ ,  $p > 0.05$ ).

## 4. Discussion

Repeated drug use has a strong effect on the brain, motivating people to reuse those substances and eventually leading to physiological and physical dependence on the drugs. Previous investigations showed that red ginseng had an inhibitory effect on the increased ambulatory activity and CPP development induced by opioid and psychostimulants [19,20]. Therefore, our finding on the effect of several doses of RGE (Fig. 1B) on behavioral dependence formation caused by repeated METH exposure is consistent with previous studies.

The human drug use pattern is directly related to voluntary intake. To mimic the compulsive aspects of addiction in humans, we used an animal model of drug SA in which an animal gave operant responses in order to gain a reward. Surprisingly, only a single treatment of RGE 40 mg/kg significantly suppressed the reinforcing effect induced by METH. This result is the first observation that RGE prevents METH reinforcement of SA. In this regard, RGE was considered to have a greater impact on reinforcement of METH SA as the requirements for a response were increased. A clinical study showed that 36% of METH users who relapsed within 1 year after treatment resumed their METH use immediately following discharge [3]. Because of this, the most important goal for relieving METH addiction is the maintenance of abstinence and prevention of relapse [21]. In this regard, our data can provide a clear evidence that RGE could modulate the excessive drug-taking behavior and uncontrollable drug-seeking behavior.

In this study, it is not easy to determine which active components of RGE's action against METH. At this point, we have thought of two possible active ingredients for METH addictive behaviors. Recently, an increasing number of studies have shown that ginsenoside can penetrate the blood-brain barrier (BBB) and directly affect the CNS. When ginseng total saponin 100 mg/kg was treated orally for 1 week, Rg1 was detected in brain tissue through HPLC analysis, and the amount was estimated to be less than 0.25 ng/ml [22]. In another study using subcutaneous administration of 12.5 mg/kg Re, the mean  $C_{max}$  was 0.56 ng/ml in cerebrospinal fluid dialysate, and it was found that even small amount of Re could regulate dopamine releasing in several brain regions [23]. These findings indicated that although only a small fraction of ginsenosides penetrated BBB, they could exhibit neuropharmacological activity in the CNS. The ginsenoside contents of RGE 20 mg/kg in our study were as follows: Rb1 159.6  $\mu$ g, Rg3 64.6  $\mu$ g, Rc 62.6  $\mu$ g, Rb2 57.8  $\mu$ g, Rg2s 44  $\mu$ g, Re 37.2  $\mu$ g, Rg1 32.6  $\mu$ g, Rf 32  $\mu$ g, Rh1 23.4  $\mu$ g, Rd 20.6  $\mu$ g. Assuming from studies mentioned above, the amount of ginsenosides that passed through the brain would be a few nanogram. Interestingly, however, it was found that RGE with

ginsenosides contents similar to our study protected the disruption of the BBB and also alleviated dopaminergic neuronal damage in the striatum of PD model [24]. It implies that a few nanogram of ginsenosides used in our study are capable of neuropharmacological activities in the brain. Therefore, it is thought that ginsenosides in RGE may exert synergistic effects together and be possible active ingredients to inhibit METH-induced addictive behaviors.

Another possible active ingredient is gintonin, which is a non-saponin polymer known as an agonist of lysophosphatidic acid receptors [25]. According to recent studies, it was shown that gintonin regulated dopamine transmission in PC12 cells and alleviated MPTP-induced motor impairments with enhancing TH level in striatum [26,27]. These suggested that gintonin exerted neuroprotective effects on dopaminergic neurons. Therefore, we suppose that gintonin in RGE may be possible active ingredients to inhibit METH-induced addictive behaviors.

In the central nervous system, METH acts as a DAT substrate, which switches the DAT binding site toward the cytosol, resulting in a large amount of DA efflux. Previous studies using DAT knockout mice showed that there was no change in amphetamine-mediated DA release in freely moving mice [28,29]. Our data showing that RGE attenuated the enhanced level of DAT induced by METH is consistent with these results, suggesting that RGE treatment may relieve the increased synaptic DA capacity. This is the first study to confirm the inhibitory effect of RGE on the activation of reversed DAT in animals with METH-seeking behavior. According to previous studies, ginseng total saponin exhibited a significant neuroprotective effect on METH-induced DA depletion [12]. In addition, *in vivo* microdialysis analyses showed that METH-induced extracellular DA release was significantly inhibited by a ginseng-related compound [30]. Collectively, we assert that RGE may preferentially inhibit the enhanced dopaminergic neurotransmission at the presynapse by regulating DAT.

In the present study, RGE blocked the increased D1DR expression in the NAc of METH RI. It is noteworthy that the study of the effect of RGE on DA receptor activity induced by METH-seeking behavior has been rare until now. A clinical study in subjects with chronic METH addiction showed a large increase in D1DR but a nonsignificant change in D2DR in the NAc; the authors commented that the increased magnitude of D1DR activation was relevant to drug-seeking behavior in the subjects [31]. Furthermore, in a rat SA test, a D1-like antagonist could inhibit the drug-seeking behavior, but a D2-like antagonist could not [32]. Optogenetic stimulation in the NAc showed that excitation of D1 neurons supported a strong seeking behavior in a self-stimulation task, but mice stimulated at D2 MSN in the NAc failed to develop a positive preference [33]. Taken together, our research is the first paper to mention that RGE prevents METH-seeking behaviors via postsynaptic D1DR regulation.

Previous studies have examined the interaction of DA and NMDAR in drug reinforcement-related learning. Parsegian and See [34] showed that METH reinstatement led to glutamate releasing in NAc. They explained that the increase of DA efflux in PFC activated the excitatory D1DR projecting to the NAc, which led a subsequent increase in glutamate releasing in NAc. Other studies revealed that the pharmacological blockade of NMDA receptor in NAc blocked the increase in DA efflux in NAc and decreased cue-induced RI of cocaine-seeking behavior [35,36]. These studies supported that reinstatement-evoked DA efflux in NAc was modulated by NMDARs function. In addition, in a study that examined which subunit composition of NMDARs was more essential in the NAc, it was found that NR2A-containing NMDARs were more necessary for DA-induced synaptic plasticity [37]. Similarly, in the present study, NMDARs, such as NR1, NR2A, and NR2B, were increased in the NAc of mice in response to METH-seeking behavior. Moreover, these

increases were reversed by RGE treatment. Taken together, these results provide evidence that RGE can modulate the function of NMDARs in NAc, with respect to an interaction with dopaminergic system, in the process of METH seeking behavior.

Currently, several lines of studies have focused on ERK-related signaling in response to addictive behaviors. When ERK activity in the NAc was inhibited, there was a dose-dependent decrease of amphetamine CPP without a deficit of motor activity [38]. Thus, in this paper, we performed Western blot analysis of the NAc of reinstated mice and analyzed the levels of D1DR downstream targets such as AC1 and PKA, followed by analysis of the levels of NMDAR-dependent CaMKII phosphorylation as well as ERK phosphorylation. In an *in vitro* study, selective DA receptor activation using a D1DR agonist induced ERK phosphorylation but not p-JNK or p-39 MAPK activation [39]. In *in vivo* study, METH-induced hyperlocomotor activity and its accompanying expression of pERK were attenuated by D1DR antagonist injection [40]. This is believed to be related to the supersensitivity of the D1DR with ERK. Actually, D1DR is generally coupled to G proteins of the G<sub>s</sub> subfamily that stimulate AC and cyclic adenosine monophosphate (cAMP) production. Friedman et al [41] found that D1DR-deficient mutant mice had reduced G<sub>s</sub>-coupled D1DR binding with decreased cAMP production. Therefore, consistent with previous studies, our data indicated that the enhanced D1DR activity by METH-primed RI induced pERK activation via the AC-PKA cascade. At the same time, we found overexpression of pCaMKII, which is considered to be due to the Ca<sup>2+</sup> influx influenced by NMDAR. In a previous study using NMDAR-knockout mice, there was a reduction of METH-induced pERK, indicating a relevance to NMDAR-mediated ERK activity [42]. Also, it is thought that the large amount of Ca<sup>2+</sup> influx further stimulates Ca<sup>2+</sup>-sensitive AC1 [43], and that the PKA-ERK cascade is more active. However, in the RGE-treated group, it was confirmed that alteration of all downstream expression could be down-regulated. Previously, it had been reported that several ginsenosides in red ginseng improved cognitive dysfunction by mediating ERK activity [44,45]. Contrary to previous papers, our study highlights the inhibitory effect of RGE on the expression level of ERK in METH-induced changes in response to METH RI.

In conclusion, the present study is very important in explaining the inhibitory effect of RGE on two primary aspects (behavioral and pharmacological), in that RGE prevents METH-induced development, reinforcement, and RI and suppresses METH-driven protein level of DAT, D1DR, NMDAR, and related intracellular signaling in the NAc. Collectively, our data may prove RGE to be a potential therapeutic candidate for METH-induced RI.

#### Author contributions

CGJ, SYL, and BRL designed the study. BRL, SJS, KHH, SEK, and SXM performed the SA test. BRL, SKK, and YJK carried out the CPP test. YKK and YL assisted with data analysis and interpretation. BRL and SJS performed Western blot. CGJ and BRL wrote the manuscript. CGJ was responsible for the overall direction of the project and for edits to the manuscript.

#### Declaration of competing interest

The authors declare that they have no competing interests.

#### Acknowledgments

This work was supported by a 2018 grant from the Korean Society of Ginseng with funding from the Korea Ginseng Corporation.

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