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Research Article

Anxiolytic effect of Korean Red Ginseng through upregulation of serotonin and GABA transmission and BDNF expression in immobilized mice

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ABSTRACT

Background: Anxiolytic properties of Korean Red Ginseng (KRG) have been previously reported. However, the exact mechanism(s) of action remains to be elucidated. The present study investigated the effect of KRG on immobilization-induced anxiety-like behaviors in mice and explored the involvement of the serotonin and GABA systems and BDNF in the anxiolytic action.

Methods: Mice were orally administered with KRG (200 mg/kg/day) for 4 weeks and immobilized once daily for 2 h. *p*-Chlorophenylalanine (*p*-CPA) was intraperitoneally injected on day 22-28, and flumazenil or bicuculline was injected on day 25-28. After behavioral evaluations, brains were dissected for biochemical analyses.

Results: KRG improved immobilization-induced anxiety-like behaviors in mice, as assessed by the elevated plus maze (EPM) and marble burying tests (MBT). The anxiolytic effect of KRG was comparable to that of fluoxetine, a reference drug clinically used for anxiety disorders. A serotonin synthesis inhibitor, *p*-CPA, blocked the effect of KRG in the EPM and MBT, indicating the requirement of serotonin synthesis for anxiolytic action. In addition, the anxiolytic effect of KRG was inhibited by bicuculline (a $GABA_A$ antagonist) in MBT, implying the involvement of GABA transmission. Western blotting analyses revealed that KRG upregulated the expression of tryptophan hydroxylase and $GABA_A$ receptor in the brain, which was blocked by *p*-CPA. Enhanced BDNF expression by KRG in the hippocampus was also indicated to mediate the anxiolytic action of KRG in immobilized mice.

Conclusion: KRG exhibited the anxiolytic effect in immobilized mice by multiple mechanisms of action, involving enhanced serotonin and GABA transmissions and BDNF expression.

1. Introduction

Anxiety disorders are the most common type of psychiatric disorders. According to large population-based surveys, anxiety affects up to 33.7% of the population during their lifetime, becoming an increasing psychoclinical challenge worldwide [1]. The current conceptualization of the etiology of anxiety includes an initial exposure to unavoidable factors like chronic stress, trauma, or a genetic vulnerability, resulting in neurobiological and neuropsychological alterations [2,3]. Despite the effectiveness of current pharmacotherapies for anxiety disorders, treatment failures may occur due to delayed responsiveness or unresponsiveness to drugs or intolerable adverse effects [4,5]. To overcome these challenges, there has been growing attention toward alternative therapeutic strategies, particularly targeting natural products or herbal medicine [6,7].

Korean Red Ginseng (KRG), produced from the roots of Korean ginseng (*Panax ginseng* Meyer, Araliaceae) cultivated for 4–6 years through repeated steaming and drying processes, has been widely used in traditional medicine for numerous neuropsychiatric disorders, including anxiety and depression [6,7]. However, only a few studies have been reported to show the anxiolytic effects of KRG in mice or rats. Red varieties of *P. ginseng* was shown to exert anxiolytic action in the open field test and elevated plus maze (EPM) test,

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and this effect was comparable to diazepam [8]. In another study, while KRG water extract did not increase the percentage of open arm entries or the time spent in open arms in the EPM test, a single oral administration of KRG butanol fraction was shown to exhibit anxiolytic-like effects in mice [9]. Moreover, KRG attenuated anxiety-like behaviors in rats during ethanol withdrawal [10]. Although few studies have identified the active constituent(s) of KRG, ginseng saponins are suggested to play important roles in anxiolytic actions [9]. For example, the crude saponin fraction and several ginsenosides from KRG, such as Rg3 and Rh2, exhibited anxiolytic effects in the EPM model [11]. Interestingly, the anxiolytic-like activities of Rg3 and Rh2 were antagonized by flumazenil (a benzodiazepine antagonist), suggesting their actions via the GABA/benzodiazepine system.

GABA is a well-characterized neurotransmitter associated with anxiety disorders [12]. The lack of inhibitory neurotransmitter GABA and its major receptor ($GABA_A$ receptors) plays important roles in the pathophysiology of anxiety, which provides pharmacological rationale to treat anxiety disorders with benzodiazepines, such as diazepam [12]. However, several associated issues, including drug dependence, rebound anxiety, and memory impairment, restrict their use to short-term treatment of acute anxiety [13]. In addition to GABA, serotonergic dysfunction has also been associated with the etiology of anxiety disorders [4,14]. Due to the favorable benefit-risk ratio, selective serotonin reuptake inhibitors, such as fluoxetine (FLX), and serotonin-norepinephrine reuptake inhibitors are recommended as first-line drugs to treat anxiety [4,15]. However, the onset of the anxiolytic effect of these drugs has a latency of 2-4 weeks after administration. Although the involvement of serotonergic system in the antidepressant effect of KRG or ginsenosides is well-characterized [16,17], its role in the anxiolytic effect is not elucidated yet.

This study aims to characterize the anxiolytic effect of KRG in a mouse model of anxiety induced by immobilization stress, using the EPM and marble burying tests (MBT). To elucidate the role of serotonin system, we investigate the impact of *p*-chlorophenylalanine (*p*-CPA, a selective 5-HT synthesis inhibitor) on the anxiolytic effect of KRG in mice. Moreover, since the modulation of GABA system has been proposed to mediate anxiolytic action of KRG [11], we also examine the effects of flumazenil and bicuculline (a competitive $GABA_A$ antagonist). Furthermore, biochemical changes mediating the serotonin and GABA systems are studied in the mouse brain. FLX is used as a positive reference drug in this study.

2. Materials and methods

2.1. Chemicals and reagents

The standardized KRG extract was manufactured from the roots of 6-year Korean ginseng by the Central Research Institute, Korea Ginseng Corporation (Daejeon, Korea), and kindly provided (Lot No. H1312-9040). According to the manufacturer's data, the main components of KRG are: ginsenosides Re (0.82 mg/g), Rf (1.37 mg/g), *(S)*-Rg2 (1.50 mg/g), Rb1 (5.85 mg/g), Rc (2.29 mg/g), Rb2 (2.17 mg/g), Rd (0.89 mg/g), *(S)*-Rg3 (4.43 mg/g), *(R)*-Rg3 (2.02 mg/g), and Rh1 (1.28 mg/g).

KRG was dissolved in distilled water to get the working solution of 20 mg/mL. FLX, flumazenil, and bicuculline were bought from Sigma-Aldrich (St. Louis, MO, USA), and *p*-CPA was purchased from Tocris (Bristol, UK). All other chemicals were of analytical grade.

2.2. Animals and experimental design

Male ICR mice (20–25 g) were obtained from Orient Bio (Gyeonggi, Korea). The animals were maintained under a controlled temperature (22 ± 2°C) and relative humidity (40–60%) with a 12-h light-dark cycle, and given free access to a standard chow diet and water. All experimental procedures including the use, care, and handling of animals were conducted following the international guidelines (Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council; National Academy Press: Washington D.C., 1996). Prior to the study, the rationale, design, and protocols of the experiments were approved by the Institutional Animal Ethical Committee of Dongguk University (approval number: IACUC-2019-001-2).

The animals were randomly allocated into seven groups of nine mice each: Vehicle-treated control group; Vehicle-treated immobilized group; FLX-treated immobilized group; KRG-treated immobilized group; KRG + *p*-CPA-treated immobilized group; KRG + flumazenil-treated immobilized group; and KRG + bicuculline-treated immobilized group.

All animals were subjected to immobilization stress during day 1–27, except for the vehicle-treated control group. Experimental drugs were administered to each group as follows: To the FLX group, FLX (10 mg/kg) were orally administered for 4 weeks. To the KRG-treated groups (KRG, KRG + *p*-CPA, KRG + flumazenil, and KRG + bicuculline), KRG (200 mg/kg) was orally administered for 4 weeks. The dosage of KRG was determined based on the previous reports [18–20]. As depicted in Fig. 1, *p*-CPA (100 mg/kg) was intraperitoneally injected on day 22–28, and flumazenil (3 mg/kg) or bicuculline (0.7 mg/kg) was injected on day 25–28, 15 min before KRG administration. The dosages, routes of administration, and duration of FLX and *p*-CPA [16], flumazenil [21], and bicuculline [22] were chosen from previous studies. Corresponding volumes of vehicle were given to the animals in control and vehicle-treated immobilized groups, instead.

To induce chronic anxiety-like behaviors, the mice were subjected to immobilization stress according to the previous reports [23,24]. Briefly, 1 to 2 h after KRG or FLX administration, mice in the immobilized groups were restrained in 50-mL conical tubes (3 cm in diameter and 10 cm in length) once daily for 2 h. A breathing hole (0.3 cm in diameter) was inserted at the end of each tube to allow air to pass directly into the nose of the mouse. Body weights were measured daily for 4 weeks. Behavioral tests, including the rota-rod test (RRT), EPM, and MBT, were then performed on day 27–28

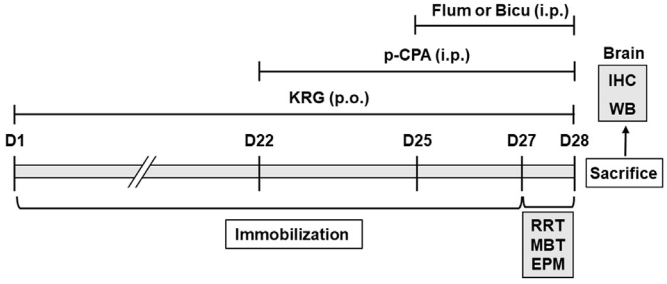


Fig. 1. Experimental schedules of immobilization, drug administration, and behavioral tests in mice. D, day; KRG, Korean Red Ginseng; *p*-CPA, *p*-chlorophenylalanine; Flum, flumazenil; Bicu, bicuculline; FLX, fluoxetine; MBT, marble burying test; EPM, elevated plus maze test; RRT, rota-rod test; IHC, immunohistochemistry; WB, western blotting.

(Fig. 1), and analyzed by investigators blinded to the treatment conditions. Following the behavioral tests, all animals were sacrificed and their brains were immediately dissected for western blotting and immunohistochemistry (IHC). The experimental schedules are collectively summarized in Fig. 1.

2.3. Rota-rod test (RRT)

RRT was performed to assess the motor coordination of animals in each group, using the rota-rod apparatus for mice (Ugo Basile Corporation, Varese, Italy), as described previously with minor modifications [25]. It was set with an accelerated velocity from 4 to 40 rpm. On the day before the test session (day 27), mice were trained to the rotating rods for 5 min at 1 h after drug administration. During the test session, endurance time (s) and the number of falls (falling frequency) were recorded for 5 min.

2.4. Elevated plus maze (EPM) test

The EPM test was performed according to the procedures reported previously with minor modifications [9,26]. The EPM apparatus is comprised of two closed arms (74 cm × 6 cm) enclosed by walls with a height of 20 cm and two open arms (74 cm × 6 cm) without walls. These arms are connected by a central (6 cm × 6 cm) square. The entire maze was elevated 50 cm above the floor. Animals were placed on the middle square facing a closed arm and allowed to explore the apparatus for 10 min. The movement of animals was recorded using a video camera-based EthoVision Maze Test System (Noldus Information Technology, Wageningen, Netherlands). Time spent in open arms or closed arms (s), number of open arm entries, and total moved distance (cm) were determined.

2.5. Marble burying test (MBT)

The MBT was performed as previously described with some modifications [27]. Briefly, clean rat cages (17 cm × 25 cm × 40 cm) were filled with wood chip bedding to a depth of 4 cm and evenly spread to a flat surface. Sixteen glass marbles were then placed at equal intervals on the bedding in a 4 × 4 grid. Mice were placed in the middle of the cage and allowed to bury marbles for 30 min without disturbance. The marbles covered with bedding up to 2/3 of the surface area were counted.

2.6. Western blotting

After behavior tests, animals were sacrificed and their brains were quickly removed. The brains were dissected to obtain the cortex and the hippocampus, which were then separately homogenized in cold lysis buffer, as previously described [28]. The homogenates were centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatants containing extracted proteins were collected and stored at −80°C until used for biochemical study. Electrophoresis and immunoblotting were then performed according to procedures described previously [29], with primary antibodies specifically recognizing tryptophan hydroxylase (TPH), glutamic acid decarboxylase with a molecular weight of 67 kDa (GAD67) (GAD1, Cell Signaling Technology, Danvers, MA, USA), $GABA_A$ receptor, and brain-derived neurotrophic factor (BDNF) (Abcam, Cambridge, MA, USA). Blots were visualized with a Bio-Rad ChemiDoc XRS imaging system using enhanced chemiluminescence reagents (Bio-Rad, Hercules, CA, USA).

2.7. Immunohistochemistry (IHC) procedure

IHC was conducted as previously described with some minor modifications [28]. Briefly, mice were perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. The brains were removed, fixed overnight, cryoprotected with 30% sucrose at 4°C, and embedded with optimum cutting temperature solution. The embedded brains were then cut with a cryostat (Leica Microsystems Ltd., Nuβloch, Germany) into 10-μm sections, mounted onto poly-L-lysine-coated slides, and surrounded by a hydrophobic barrier with a wax pen (ImmEdge Hydrophobic Barrier PAP Pen, Vector Laboratories, Burlingame, CA, USA). Nonspecific binding was blocked with 5% goat serum and 0.5% Triton X-100 in PBS for 1 h at room temperature. The sections were incubated with anti-BDNF antibody (1:100 dilution) overnight at 4°C. After washing with PBS three times, the sections were incubated with fluorescent-conjugated secondary antibody (1:400 dilution) at room temperature for 1 h in the dark and mounted with anti-fade mounting medium containing DAPI. Fluorescence was visualized using a Nikon confocal laser-scanning microscope (Nikon Instruments Inc., Melville, NY, USA).

2.8. Statistical analysis

The quantitative data were presented as mean ± SEM. Statistical analysis was conducted by one-way ANOVA, followed by Tukey's post hoc test using SigmaPlot 12.5 software (Systat Software Inc., San Jose, CA, USA). A $p < 0.05$ was considered significant.

3. Results

3.1. Effect of immobilization-induced chronic stress on body weight of mice

The body weights of mice were measured daily for 28 days. All animals gained body weight during 28 days of the experiment. However, the weight gain of animals in the immobilized groups was significantly less than that in the control group, starting from day 4. Neither KRG nor FLX restored the reduced rate of weight gain by immobilization. In addition, *p*-CPA, flumazenil, or bicuculline administration along with KRG did not significantly change the body weights of the immobilized mice (Fig. 2A).

3.2. Effect of experimental drugs on motor coordination in immobilized mice

Prior to investigating the effect of KRG on the immobilization-induced anxiety-like behaviors and its underlying mechanism(s), RRT was carried out to examine whether our experimental drugs, including KRG, could influence motor coordination in mice. The falling frequency in the control group appeared to be relative high, probably due to the short training period [30]. However, there were no significant changes in endurance time and falling frequency in all animal groups (Fig. 2B), indicating that our experimental drugs did not affect motor coordination and balance. Behavioral studies, including the EPM and MBT, were then conducted to evaluate the effect of KRG on the immobilization-induced anxiety-like behaviors.

3.3. Effect of KRG on immobilization-induced anxiety-like behaviors in the EPM test

The EPM test is commonly used to evaluate the effects of drugs on exploratory behaviors and anxiety in rodents. Increased open arm entries and time spent in the open arms indicate anxiolytic

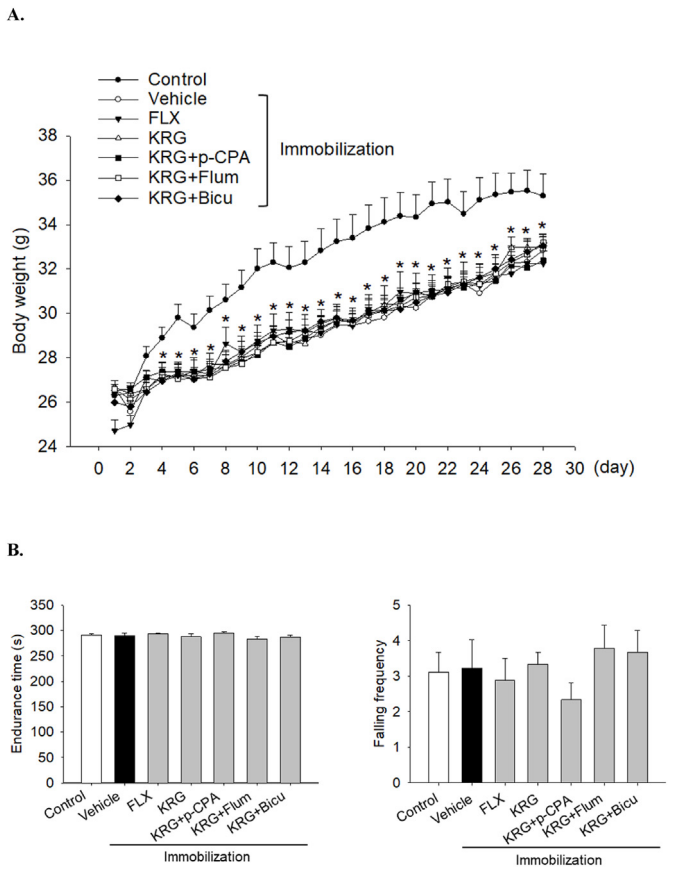


Fig. 2. Effects of KRG and experimental drugs on body weight and motor coordination in mice. (**A**) Body weights were measured daily for 4 weeks and presented as mean ± SEM. *$p < 0.05$ vs. vehicle-treated control group (n = 9 per group). (**B**) Rotarod test was performed as described in the Materials and methods. Endurance time (left) and falling frequency (right) are presented as the mean ± SEM (n = 9). KRG, Korean Red Ginseng; FLX, fluoxetine; *p*-CPA, *p*-chlorophenylalanine; Flum, flumazenil; Bicu, bicuculline.

activities in the test [26,31]. To evaluate the anxiolytic effect of KRG, anxiety-like behaviors were induced in mice by immobilization stress, and EPM tests were performed after oral administration of KRG (200 mg/kg) for 4 weeks.

In the EPM test, the time spent in open arms, the time spent in close arms, the number of open arm entries, and the total moved distance were determined (Fig. 3). The time spent in open arms was markedly reduced in the vehicle-treated immobilized group (Fig. 3A), indicating that immobilization successfully induced anxiety-like behaviors. While the number of entries to open arms in this group also showed a tendency to decrease (Fig. 3C), the time spent in close arms was slightly increased (Fig. 3B). However, the differences were not statistically significant from those in the non-immobilized control group (Fig. 3B and C). KRG markedly reversed the reduced time spent in open arms, the increased time spent in close arms, and the decreased number of open arm entries in the immobilized mice (Fig. 3A–C), demonstrating its anxiolytic effect. Similar effects were produced by FLX (10 mg/kg), a positive reference drug, confirming its anxiolytic effect in this study (Fig. 3A–C). By contrast, the total moved distance in the EPM test was not significantly altered in all experimental groups, including the vehicle-treated immobilized group (Fig. 3D). Thus, the induction of anxiety-like behaviors by immobilization stress was not attributed to the alterations in locomotor activities of mice. As shown in the representative tracking maps (Fig. 3E), the movement in open arms (red color) of vehicle-treated immobilized mice was dramatically reduced compared to that of the non-immobilized control group. In agreement with our findings shown in Fig. 3A–C, the immobilization-induced reduction of the movement in open arms was noticeably reversed by KRG or FLX (Fig. 3E). Collectively, these results demonstrated that KRG and FLX orally administered for 4 weeks have anxiolytic effects in the mice exposed to chronic immobilization stress.

3.4. Involvement of serotonin synthesis in the anxiolytic effect of KRG in the EPM test

To examine whether the anxiolytic effect of KRG was mediated by serotonergic and/or GABAergic transmissions, the KRG-treated immobilized mice were intraperitoneally injected with a serotonin synthesis inhibitor (*p*-CPA, 100 mg/kg), a benzodiazepine antagonist (flumazenil, 3 mg/kg), or a competitive $GABA_A$ receptor antagonist (bicuculline, 0.7 mg/kg), as illustrated in Fig. 1, and EPM tests were performed. The anxiolytic effect of KRG was notably inhibited by *p*-CPA, reducing the time spent in open arms and the number of open arm entries (Fig. 3A, C, and E). However, the administration of flumazenil or bicuculline did not inhibit the anxiolytic effect of KRG in this study. These results indicate that serotonin synthesis is required for the anxiolytic effect of KRG. By contrast, the anxiolytic action of KRG evaluated by the EPM test appears not to be mediated by GABA transmission.

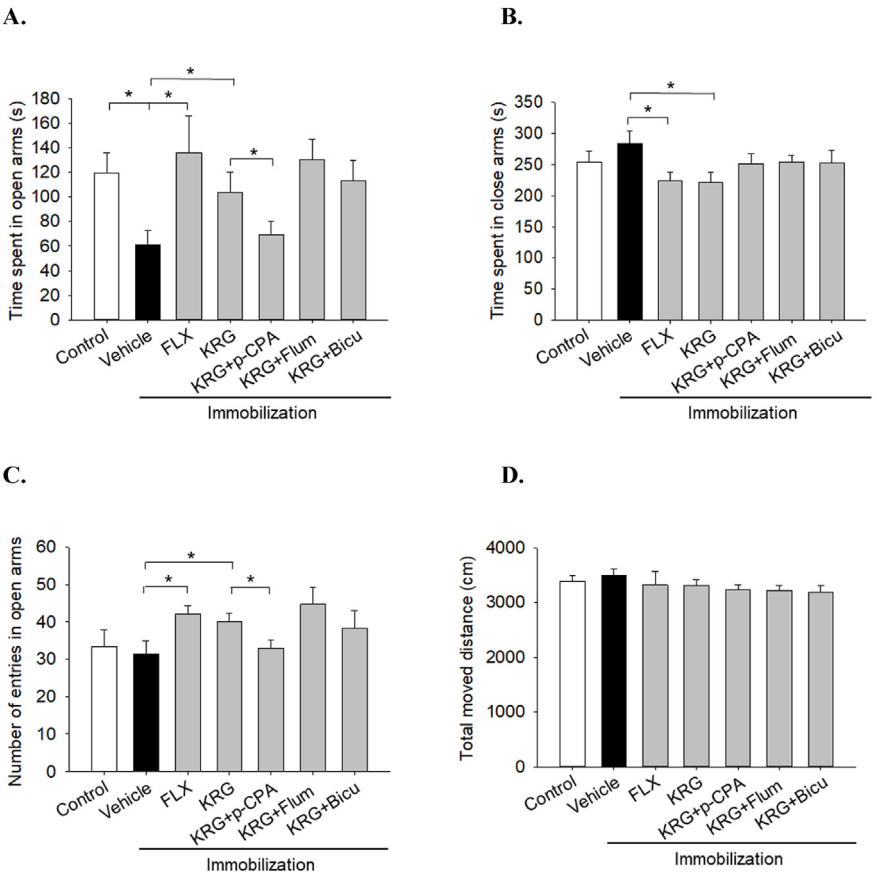
3.5. Effect of KRG on immobilization-induced anxiety-like behaviors in the MBT

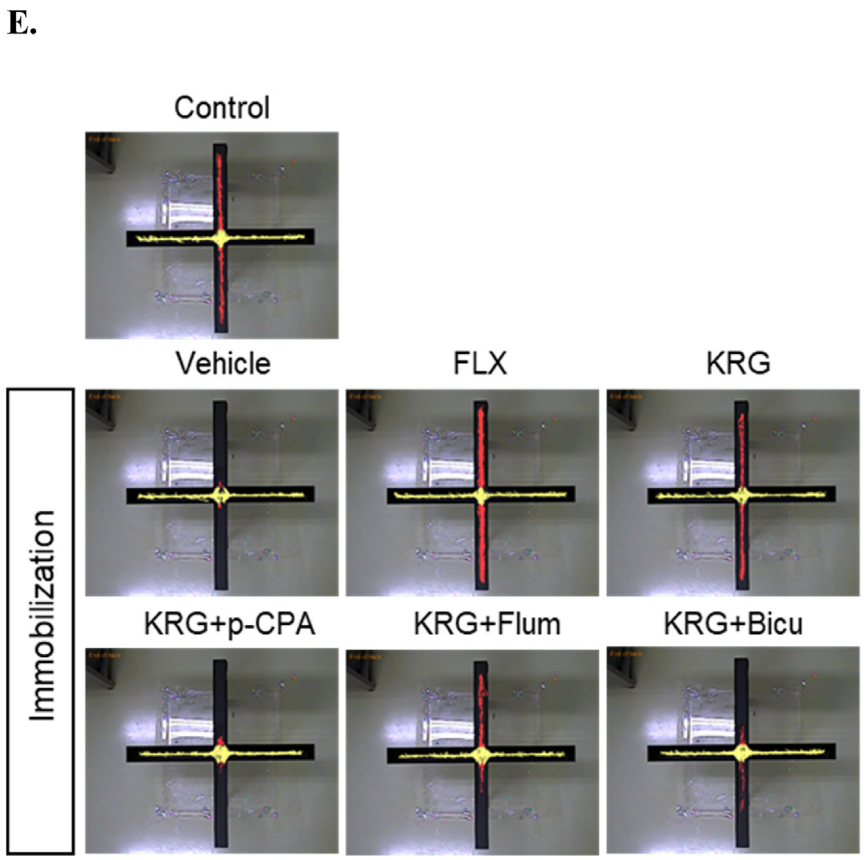
To confirm our findings, we performed MBT, another behavioral test frequently used to measure anxiety-like behaviors in mice [27]. Consistent with the previous report [32], the vehicle-treated immobilized mice showed a marked increase in the number of buried marbles, an indicator of anxiety-like behaviors in MBT (Fig. 4A, black bar; Fig. 4B). This impact was dramatically reversed in mice by orally administered KRG for 4 weeks, demonstrating the anxiolytic effect of KRG in MBT. As expected, FLX also exhibited the anti-anxiety effect (Fig. 4A and B).

The involvement of serotonergic and/or GABAergic transmissions in the anxiolytic action of KRG was also evaluated in MBT. Similar to the findings from the EPM test, *p*-CPA treatment completely blocked the anxiolytic effect of KRG in MBT (Fig. 4), revalidating the critical role of serotonin synthesis in the anxiolytic action. Interestingly, the bicuculline-treated group showed a remarkable increase in the number of buried marbles, suggesting that GABA transmission was also involved in the KRG effect. Although the number of buried marbles was also increased by flumazenil, this effect was not statistically significant (Fig. 4A).

3.6. Effects of KRG on the expressions of TPH, GAD67, and $GABA_A$ receptor in the brains of immobilized mice

Our behavioral studies using the EPM and MBT showed that *p*-CPA markedly abolished the anxiolytic effect of KRG (Figs. 3 and 4), indicating the requirement of serotonin synthesis for its action. These findings prompted us to examine whether KRG altered the expression of TPH, an enzyme that catalyzes the initial and rate-limiting step in the synthesis of serotonin, in the brains of immobilized mice. We found that the levels of TPH in the cortex and hippocampus of the vehicle-treated immobilized mice were comparable to those of non-immobilized control mice (Fig. 5A and B). However, the TPH expression in the cortex was substantially increased by KRG administration, which was completely abolished by *p*-CPA, flumazenil, or bicuculline (Fig. 5A).


A.
Time spent in open arms (s)
0
20
40
60
80
100
120
140
160
180
Control
Vehicle
FLX
KRG
KRG+p-CPA
KRG+Flum
KRG+Bicu
Immobilization
B.
Time spent in close arms (s)
0
50
100
150
200
250
300
350
Control
Vehicle
FLX
KRG
KRG+p-CPA
KRG+Flum
KRG+Bicu
Immobilization
C.
Number of entries in open arms
0
10
20
30
40
50
60
Control
Vehicle
FLX
KRG
KRG+p-CPA
KRG+Flum
KRG+Bicu
Immobilization
D.
Total moved distance (cm)
0
1000
2000
3000
4000
Control
Vehicle
FLX
KRG
KRG+p-CPA
KRG+Flum
KRG+Bicu
Immobilization


E.
Control
Vehicle
FLX
KRG
KRG+p-CPA
KRG+Flum
KRG+Bicu
Immobilization

Although both flumazenil and bicuculline failed to block the anxiolytic effect of KRG in the EPM test, bicuculline was found to significantly attenuate the KRG effect in MBT (Fig. 4A), suggesting potential involvement of GABA transmission. To test this possibility, we examined whether KRG altered the levels of GAD67 (the predominant isoform of GAD in the brain, catalyzing the decarboxylation of glutamate to synthesize GABA) and $GABA_A$ receptor in the brains of immobilized mice. While the level of GAD67 was not altered in the cortex of the vehicle-treated immobilized mice, its expression was reduced in the hippocampus compared to the control group (Fig. 5). Interestingly, both KRG and FLX increased the GAD67 expression in the cortex (Fig. 5A). However, it was not significantly altered in the hippocampus by any experimental drug tested in this study (Fig. 5B).

The levels of $GABA_A$ expression were considerably downregulated by immobilization stress in the cortex and hippocampus. The reduced cortical $GABA_A$ receptor expression was completely restored by KRG or FLX (Fig. 5A). The KRG-induced restoration of $GABA_A$ was notably inhibited by *p*-CPA, but not by flumazenil or bicuculline.

Collectively, these results indicate that enhanced serotonergic and GABAergic transmissions mediate the anxiolytic effect of KRG, mainly through upregulation of TPH and $GABA_A$ expression in the brain. Given that the upregulated TPH and $GABA_A$ receptor by KRG are distinctly inhibited by *p*-CPA, serotonin synthesis may be a crucial process to exert the anxiolytic action.

3.7. Effect of KRG on BDNF expression in the brains of immobilized mice

BDNF, a member of the neurotrophin family, plays an important role in stress-related mental disorders, such as anxiety and depression [33]. The BDNF expression is significantly affected by stress in specific brain regions. Accumulating evidence revealed that stress-associated anxiety-like phenotypes were closely related to the reduced BDNF level in the hippocampus [33]. Therefore, we evaluated the effects of KRG on the expression of BDNF in the hippocampus. In accordance with the previous reports, we also observed in western blotting analysis that the immobilization stress markedly decreased BDNF expression in the hippocampus, as noticed by the reduced intensity of band at 14 kDa corresponding to the mature form of BDNF (Fig. 6A). The reduced BDNF expression was notably upregulated by KRG or FLX. The KRG-induced upregulation of hippocampal BDNF was significantly reduced by *p*-CPA. Flumazenil or bicuculline also showed a tendency to attenuate the upregulated hippocampal BDNF, but the effect was not statistically significant. These findings were exactly reproduced in hippocampal slices by IHC analysis (Fig. 6B). Although the BDNF level in the cortex of immobilized mice was slightly decreased, the cortical BDNF expressions were not significantly altered by the experimental drugs tested in this study (Fig. 6A). Based on our findings, the upregulated BDNF expression by KRG in the hippocampus is also associated with the anxiolytic effect of KRG in immobilized mice.

4. Discussion

Beneficial effects of *P. ginseng* and its constituents have been reported in various neuropsychological conditions, such as learning and memory deficits, mental illnesses, and cerebral ischemia [34–36]. In addition, the antidepressant and anti-anxiety effects of KRG and selected ginsenosides have been documented in depressive patients and various animal models [8,11,28,37]. Multiple mechanisms have been suggested to mediate their antidepressant activities, including increases in the levels of serotonin, dopamine, and norepinephrine, upregulation of BDNF, and modulation of the hypothalamic–pituitary–adrenal axis [38,39]. By contrast, the GABA/benzodiazepine system has been proposed to be the major mechanism mediating the anti-anxiety effects of KRG and selected ginsenosides, including Rg3, Rh2, and Rg1 [11,40,41]. Although several studies have emphasized involvement of the serotonergic system in the antidepressant effect of KRG [16,17], its role in the anxiolytic effect remains to be further delineated.

The anxiolytic effects of KRG and ginsenosides have been mostly studied using naïve mice and rat models of post-traumatic stress disorder or ethanol withdrawal [8,10,41,42]. The present study evaluated the anxiolytic effect of KRG using a mouse model of anxiety induced by chronic immobilization stress. In addition, to elucidate underlying action mechanisms(s) of KRG, we explored involvement of the serotonin and GABA systems and the BDNF expression in the anxiolytic action. Considering that current pharmacotherapy to treat anxiety disorders usually requires long-term treatment, and that it usually takes at least 2–4 weeks of drug administration to exert their efficacy [4,5], we designed our experimental protocol with the duration of KRG administration for 4 weeks in this study (Fig. 1).

It is well-recognized that body weight changes are closely associated with anxiety [43]. Thus, prior to investigating the effect of KRG on immobilization-induced anxiety-like behaviors in mice, body weight changes were monitored for 28 days of our experiment. We found that immobilization stress reduced the rate of weight gain, which was not restored by the administration of any experimental drugs tested in this study (Fig. 2A). We also examined the motor coordination of animals in all groups by RRT on day 27, and found that our experimental drugs did not affect the motor coordination and balance (Fig. 2B).

We then conducted behavioral studies to evaluate the anxiolytic effect of KRG on the immobilization-induced anxiety-like behaviors. Exploration-based models, including the EPM and MBT, are commonly utilized in a majority of rodent studies to characterize behavioral alterations in animals exposed to anxious stimuli, such as fear-induced avoidance [26,27,44]. In our study, immobilization stress-induced anxiety-like behaviors were evident from the marked reduction of time spent in open arms in the EPM test and the increased number of buried marbles in the MBT (Figs. 3 and 4). Administration of KRG dramatically restored the time spent in open arms as well as closed arms and the number of open arm entries in the EPM test. Similarly, KRG effectively reversed the number of buried marbles in MBT, collectively demonstrating the anxiolytic effect of KRG. The effect of KRG was comparable to that of FLX (Figs. 3 and 4), a well-known drug clinically used for the treatment of various types of anxiety disorders, such as panic disorder, generalized anxiety disorder, and obsessive-compulsive disorder [4,5]. Our findings ascertaining the anxiolytic effects of KRG and FLX in the immobilized mice are consistent with the previous findings examined in different animal models of anxiety [10,28].

One of the most widely acknowledged hallmarks associated with the pathophysiology of anxiety is the declines in neurotransmitters, such as GABA, serotonin, and dopamine [12,45], which may cause dysfunction of their receptors, resulting in

Fig. 3. Effects of KRG and experimental drugs on immobilization-induced anxiety-like behaviors in the EPM test. EPM test was performed as described in the Materials and methods. Time spent in open arms (**A**), time spent in close arms (**B**), number of open arm entries (**C**), and total moved distance (**D**) are presented as the mean ± SEM (n = 9). *$p < 0.05$. Representative tracking maps are shown (**E**). Red-colored lines indicate the movement of mice in each experimental group. KRG, Korean Red Ginseng; FLX, fluoxetine; *p*-CPA, *p*-chlorophenylalanine; Flum, flumazenil; Bicu, bicuculline.

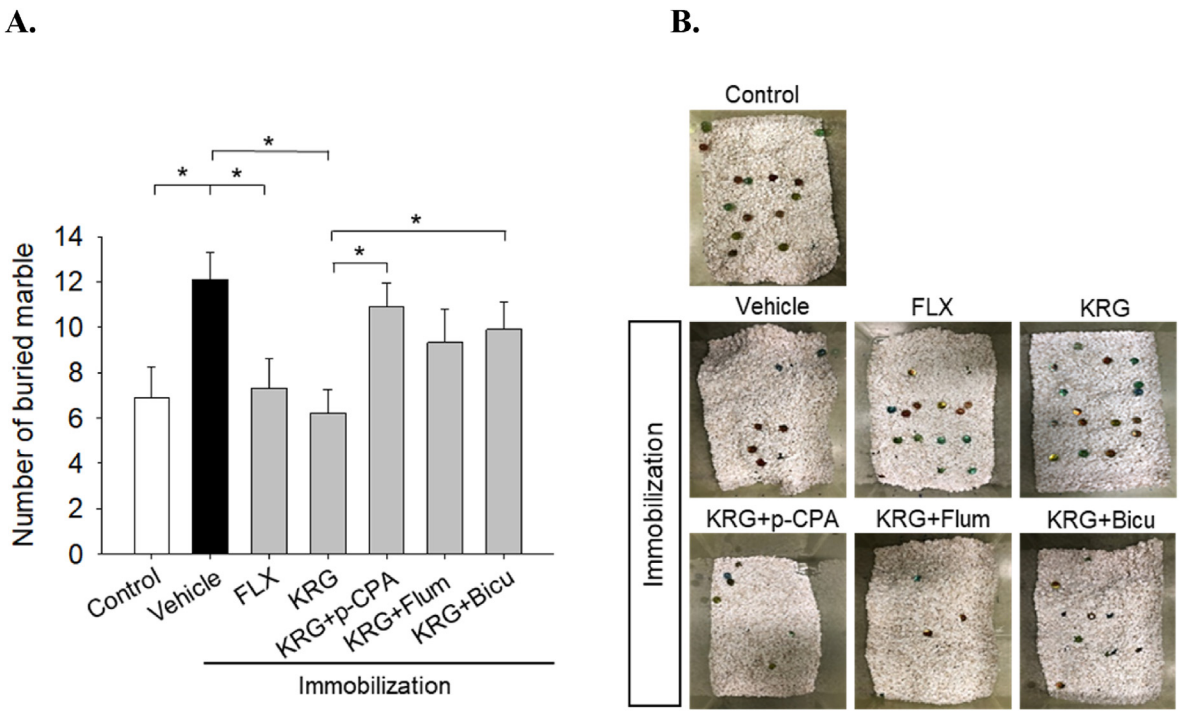


Fig. 4. Effects of KRG and experimental drugs on immobilization-induced anxiety-like behaviors in MBT. MBT was performed as described in the Materials and methods. (**A**) The number of buried marbles is presented as the mean ± SEM (n = 9). *$p < 0.05$. (**B**) Representative pictures are shown. KRG, Korean Red Ginseng; FLX, fluoxetine; *p*-CPA, *p*-chlorophenylalanine; Flum, flumazenil; Bicu, bicuculline.

impaired transmission in the brain. To establish involvement of the serotonin system in the anxiolytic effect of KRG, we used a serotonin synthesis inhibitor, *p*-CPA. As shown in Figs. 3 and 4, we found that intraperitoneally injected *p*-CPA completely abolished the anxiolytic effect of KRG in the EMP and MBT, demonstrating the crucial role of serotonin synthesis. Serotonin is synthesized from tryptophan in two steps, with TPH as the rate-limiting enzyme. Thus, we then examined whether KRG altered the expression of TPH in the brains of immobilized mice. Upon KRG administration, confirmatory evidence of a substantially increased TPH level in the cortex was observed, and this upregulation was completely abolished by p-CPA (Fig. 5A). These results suggest that KRG enhances serotonin synthesis through upregulation of TPH, which contributes to its anxiolytic effect. To examine whether KRG increased serotonin levels in the brain, we measured the level of serotonin in the cortical homogenates using an ELISA kit (Abcam, Cat. No. ab133053). As expected, the serotonin level was considerably decreased in the brain of immobilized mice, and the decreased serotonin level was dramatically recovered by KRG or FLX to the level of control group (data not shown). Direct measurement of 5-HT levels in the mouse brain could strengthen this finding. Among the serotonin receptor subtypes identified, 5-HT2A and 5-HT1A receptors are reported to be most closely related to anxiety-like behavior in post-traumatic stress disorder in mice [46]. Thus, it would be interesting to identify subtypes of the receptor mediating the effect of KRG in immobilized mice. Although KRG has been reported to prevent depression-like behaviors in post-traumatic stress disorder through enhancing 5-HT concentration in the hippocampus [17], our study is the first to elucidate the requirement of serotonin synthesis for the anxiolytic action of KRG.

Apart from the serotonin system, we also investigated the role of GABA transmission in the anxiolytic effect of KRG. While the administration of flumazenil or bicuculline did not counteract the anxiolytic effect of KRG in our EPM test (Fig. 3), the bicuculline-treated group showed a remarkable increase in the number of buried marbles in the MBT (Fig. 4), suggesting that the GABA system might be involved in the KRG effect. The GABA/benzodiazepine system has been previously reported to mediate anxiolytic-like activities of KRG and several ginsenosides, such as Rg3, Rh2, Rb1, and Rg1 in the EPM test [11,40,41]. The discrepancy between our results and previous findings in the EPM test might be, at least in part, due to the differences in animal models of anxiety. While we used the anxiety-like animal model induced by chronic immobilization stress, previous studies used naïve animals to test the anxiolytic effects [11,40,41]. An additional investigation should be conducted to further clarify the discrepancy.

To examine the potential involvement of the GABA system, we further examined the effect of KRG on the expressions of GAD67 and $GABA_A$ in the brains of immobilized mice. We found that KRG and FLX increased the cortical GAD67 expression. However, the level of hippocampal GAD67 was not altered by KRG or FLX, compared to that of the control (Fig. 5). It remains to be determined whether the increased levels of GAD67 by KRG or FLX in the cortex play any role in their anxiolytic effects. Unlike GAD67, the levels of $GABA_A$ expression in the cortex and hippocampus were considerably downregulated by immobilization stress, and these levels were completely restored by KRG or FLX (Fig. 5). Collectively, our data demonstrate that KRG exerts its anxiolytic effect by enhancing serotonin transmission through upregulation of TPH and restoring

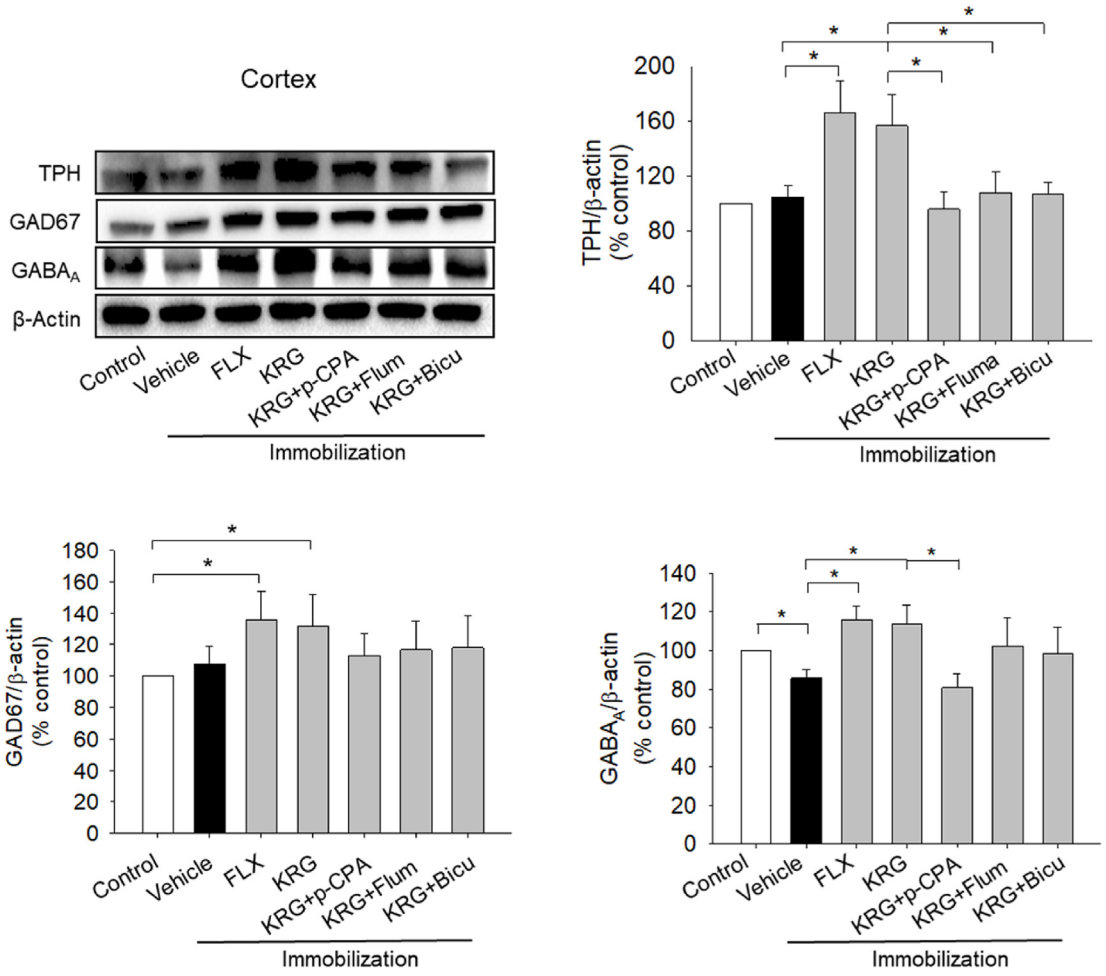


Fig. 5. Effects of KRG and experimental drugs on the expressions of TPH, GAD67, and $GABA_A$ receptor in the brains of immobilized mice. The brains were collected and the cortex and hippocampus were dissected. Western blotting analyses of the cortical (**A**) and hippocampal (**B**) lysates were performed using antibodies specifically recognizing TPH, GAD67, and $GABA_A$ receptor, respectively. Representative bands are shown. Data are presented as the mean ± SEM from three independent experiments. *$p < 0.05$. KRG, Korean Red Ginseng; FLX, fluoxetine; *p*-CPA, *p*-chlorophenylalanine; Flum, flumazenil; Bicu, bicuculline; TPH, tryptophan hydroxylase; GAD67, glutamic acid decarboxylase with a molecular weight of 67 kDa; GABA, γ-aminobutyric acid.

the disrupted GABA transmission through upregulation of $GABA_A$ receptors in the immobilized mice.

Interestingly, our findings imply that there may be mutual interactions between the serotonin and GABA systems to mediate the anxiolytic effect of KRG, as *p*-CPA and flumazenil or bicuculline reduced the expression of $GABA_A$ and TPH, respectively (Fig. 5). In support of this finding, direct interactions between the GABA and serotonin systems in the raphe nuclei and cortical regions have been reported [47,48]. A low basal level of GABA may result in the reduced facilitation of serotoninergic transmission in mood disorders [49] and suppression of serotonin activity is likely to disinhibit benzodiazepine behavior, manifesting anxiolytic actions [50].

Besides serotonin and GABA, other neurotransmitters have also been reported to be involved in the anxiolytic action of KRG. For example, Zhao et al [10] reported that KRG attenuated anxiety-like behaviors in rats during ethanol withdrawal through enhanced mesoamygdaloid dopamine system. Moreover, oral administration of ginsenoside Rb1 once daily for 14 consecutive days suppressed anxiety-like responses in a rat model of post-traumatic stress disorders, possibly through the modification of hypothalamic neuropeptide Y expression, tyrosine hydroxylase expression in the locus coeruleus, and hippocampal mRNA expression of BDNF [28]. To test the potential involvement of BDNF expression in the anxiolytic effect of KRG in immobilized mice, we also studied alterations in BDNF expression in the hippocampus. Our results showed that immobilization significantly reduced the mature BDNF levels in the hippocampus, but not in the cortex, and the reduced BDNF was effectively normalized by KRG (Fig. 6).

It has been demonstrated that exposure to chronic stress abate mRNA and protein expression of BDNF in the limbic system, including the hippocampus [51,52]. Serum BDNF levels are also altered by several antidepressant drugs, such as FLX [52]. The serotonin and GABA systems and BDNF share common features of their abilities to regulate the development and plasticity of neural

B.

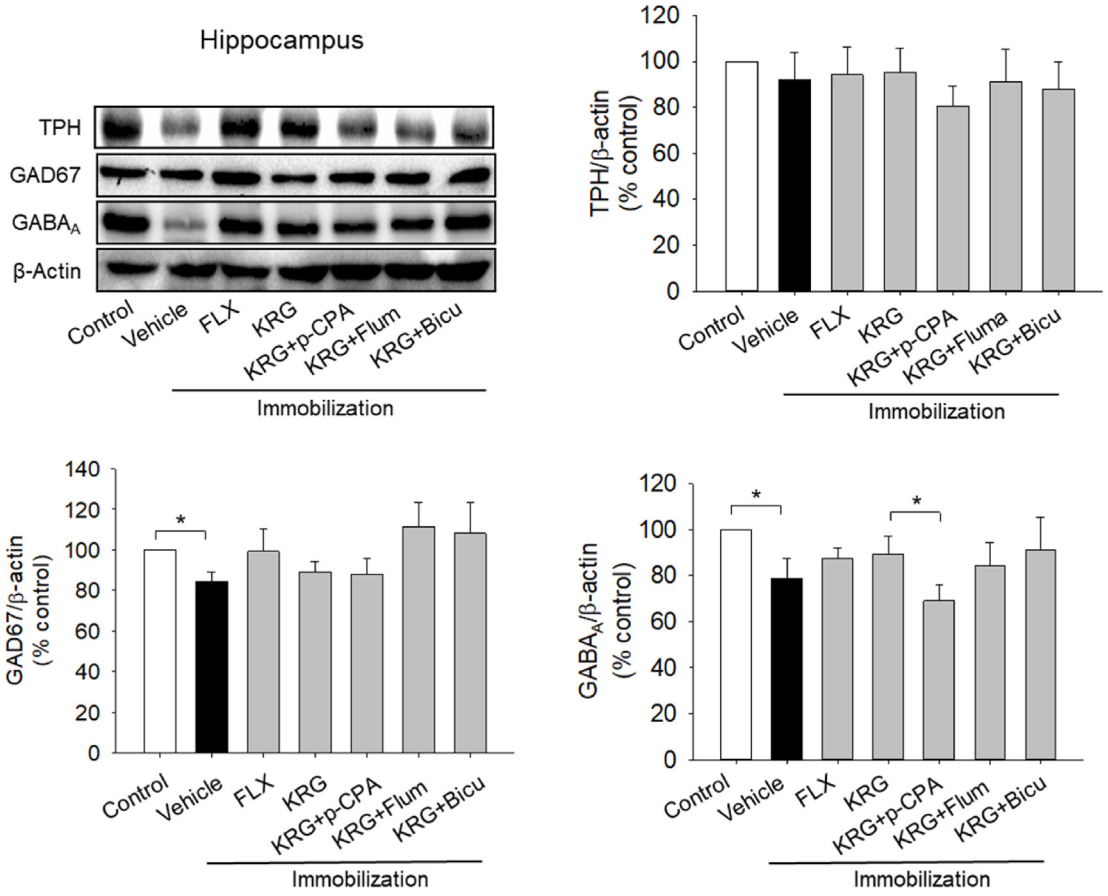


Fig. 5. (*continued*).

circuits involved in mood disorders, including depression and anxiety [53,54]. Our data revealed that *p*-CPA, flumazenil, or bicuculline dramatically inhibited the BDNF level elevated by KRG (Fig. 6), implying that enhanced BDNF expression in the hippocampus may be mediated by both serotonergic and GABAergic transmissions in the immobilized mice. Reciprocal interactions between BDNF and serotonin system have been demonstrated, in which BDNF promotes the development and function of serotonergic neurons expressing TrkB, the high-affinity receptor for mature BDNF [55]. The enhanced serotonergic transmission has been suggested to increase the production of cAMP, subsequently activating protein kinase A (PKA). The phosphorylation of cAMP response element-binding protein (CREB) by PKA may positively regulate the transcription of BDNF [56]. Further studies are in progress to examine whether the CREB-mediated signaling participates in the KRG-induced BDNF expression. The amygdala is another critical brain region involved in various types of stress-associated mental disorders like anxiety [57]. It would also be valuable to elucidate the effect of KRG on the BDNF expression in the amygdala of immobilized mice.

5. Conclusions

In summary, we demonstrated the anxiolytic effect of KRG in immobilized mice. Administration of KRG for 4 weeks markedly ameliorated immobilization-induced anxiety-like behaviors in mice, as assessed by the EPM and MBT. We also demonstrated that the serotonin and GABA systems and hippocampal BDNF expression were involved in the anxiolytic action. Upregulation of TPH and GABA$_A$ receptor expression by KRG may contribute, at least in part, to enhance the serotonin and GABA transmissions, respectively, in the brain. Interestingly, our data imply that the augmentation of BDNF level in the hippocampus may be associated with the enhanced serotonergic and GABAergic transmissions by KRG in the immobilized mice. Our findings would bring up new motives for elucidation of active compound(s) in KRG and molecular mechanisms underlying its anxiolytic effect. More efforts are required to clarify the connections between the serotonin and GABA systems and hippocampal BDNF expression. Collectively, KRG exhibited anxiolytic effect in the immobilized mice by multiple mechanisms of action, involving enhanced serotonin and GABA transmissions and BDNF expression. Considering the long-term

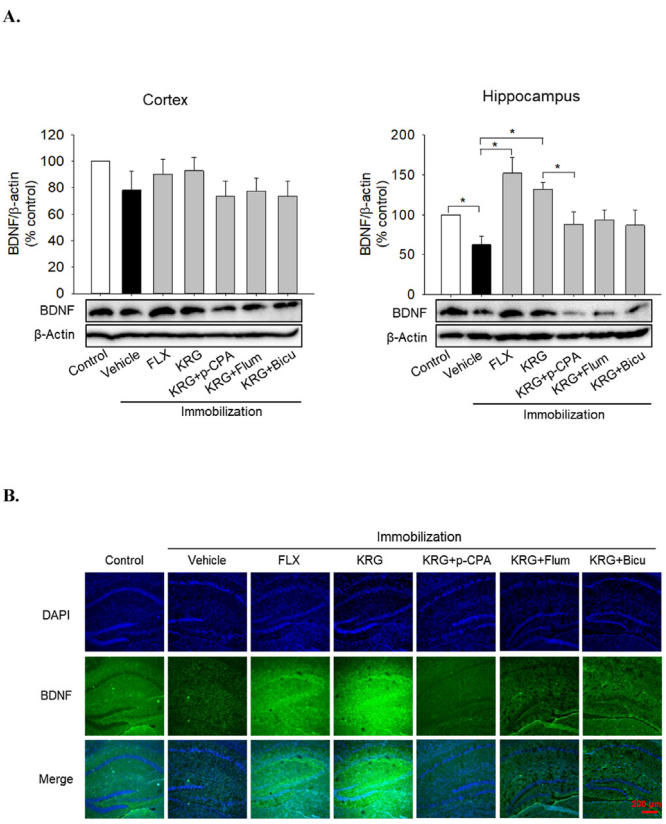


Fig. 6. Effects of KRG and experimental drugs on BDNF expression in the brains of immobilized mice. The brains of mice were collected and the cortex and hippocampus were dissected. (**A**) Western blotting analyses of the cortical and hippocampal lysates were performed using antibodies specifically recognizing BDNF. Quantitative analyses of the band were carried out. Data are presented as the mean ± SEM from three independent experiments. *$p < 0.05$. Representative blots are shown. (**B**) The brains of mice were removed in each group and cut into 10-μm sections using a cryostat. Immunohistochemical study was performed using anti-BDNF antibody and visualized using a Nikon confocal laser-scanning microscope as described in the Materials and methods. Representative immunofluorescence images are shown. Scale bar, 200 μm. KRG, Korean Red Ginseng; FLX, fluoxetine; *p*-CPA, *p*-chlorophenylalanine; Flum, flumazenil; Bicu, bicuculline; BDNF, brain-derived neurotrophic factor.

safety profile of KRG, it may offer a promising alternative for anxiety treatment.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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References

[1] Bandelow B, Michaelis S. Epidemiology of anxiety disorders in the 21st century. Dialogues Clin Neurosci 2015;17:327–35.
[2] Gottschalk MG, Domschke K. Genetics of generalized anxiety disorder and related traits. Dialogues Clin Neurosci 2017;19:159–68.
[3] Viggiano A, Cacciola G, Widmer DA, Viggiano D. Anxiety as a neurodevelopmental disorder in a neuronal subpopulation: evidence from gene expression data. Psychiatry Res 2015;228:729–40.
[4] Bandelow B, Michaelis S, Wedekind D. Treatment of anxiety disorders. Dialogues Clin Neurosci 2017;19:93–107.
[5] Blanco C, Raza MS, Schneier FR, Liebowitz MR. The evidence-based pharmacological treatment of social anxiety disorder. Int J Neuropsychopharmacol 2003;6:427–42.
[6] Jeong HG, Ko YH, Oh SY, Han C, Kim T, Joe SH. Effect of Korean Red Ginseng as an adjuvant treatment for women with residual symptoms of major depression. Asia Pac Psychiatry 2015;7:330–6.
[7] Kim Y, Cho SH. The effect of ginsenosides on depression in preclinical studies: a systematic review and meta-analysis. J Ginseng Res 2021;45:420–32.
[8] Bhattacharya SK, Mitra SK. Anxiolytic activity of *Panax ginseng* roots: an experimental study. J Ethnopharmacol 1991;34:87–92.
[9] Park JH, Cha HY, Seo JJ, Hong JT, Han K, Oh KW. Anxiolytic-like effects of ginseng in the elevated plus-maze model: comparison of red ginseng and sun ginseng. Prog Neuropsychopharmacol Biol Psychiatry 2005;29:895–900.
[10] Zhao Z, Kim YW, Wu Y, Zhang J, Lee JH, Li X, Cho IJ, Park SM, Jung DH, Yang CH, et al. Korean Red Ginseng attenuates anxiety-like behavior during ethanol withdrawal in rats. J Ginseng Res 2014;38:256–63.
[11] Kim TW, Choi HJ, Kim NJ, Kim DH. Anxiolytic-like effects of ginsenosides Rg3 and Rh2 from red ginseng in the elevated plus-maze model. Planta Med 2009;75:836–9.
[12] Möhler H. The GABA system in anxiety and depression and its therapeutic potential. Neuropharmacology 2012;62:42–53.
[13] Lader M. Benzodiazepine harm: how can it be reduced? Br J Clin Pharmacol 2014;77:295–301.
[14] Żmudzka E, Sałaciak K, Sapa J, Pytka K. Serotonin receptors in depression and anxiety: insights from animal studies. Life Sci 2018;210:106–24.
[15] Graeff FG, Guimarães FS, De Andrade TG, Deakin JF. Role of 5-HT in stress, anxiety, and depression. Pharmacol Biochem Behav 1996;54:129–41.
[16] Wang GL, He ZM, Zhu HY, Gao YG, Zhao Y, Yang H, Zhang LX. Involvement of serotonergic, noradrenergic and dopaminergic systems in the antidepressant-like effect of ginsenoside Rb1, a major active ingredient of Panax ginseng. *C.A. Meyer*. J Ethnopharmacol. 2017;204:118–24.
[17] Lee B, Sur B, Lee H, Oh S. Korean Red Ginseng prevents posttraumatic stress disorder-triggered depression-like behaviors in rats via activation of the serotonergic system. J Ginseng Res 2020;44:644–54.
[18] Lim SW, Doh KC, Jin L, Jin J, Piao SG, Heo SB, Chung BH, Yang CW. Ginseng treatment attenuates autophagic cell death in chronic cyclosporine nephropathy. Nephrology (Carlton). 2014;19:490–9.
[19] Lee MR, Begum S, Sung CK. Effect of red and black ginseng on cholinergic markers, presynaptic markers, and neurotrophins in the brain of aged mice. Food Sci Biotechnol 2017;26:1743–7.
[20] Gonzales EL, Jang JH, Mabunga DF, Kim JW, Ko MJ, Cho KS, Bahn GH, Hong M, Ryu JH, Kim HJ, et al. Supplementation of Korean Red Ginseng improves behavior deviations in animal models of autism. Food Nutr Res 2016;60:29245.
[21] Abouhosseini Tabari M, Hajizadeh Moghaddam A, Maggi F, Benelli G. Anxiolytic and antidepressant activities of *Pelargonium roseum* essential oil on Swiss albino mice: possible involvement of serotonergic transmission. Phytother Res 2018;32:1014–22.
[22] Alonso-Castro AJ, Gasca-Martínez D, Cortez-Mendoza LV, Alba-Betancourt C, Ruiz-Padilla AJ, Zapata-Morales JR. Evaluation of the neuropharmacological effects of *Gardenin A* in mice. Drug Dev Res 2020;81:600–8.
[23] Jung JM, Park SJ, Lee YW, Lee HE, Hong SI, Lew JH, Hong E, Shim JS, Cheong JH, Ryu JH. The effects of a standardized *Acanthopanax koreanum* extract on stress-induced behavioral alterations in mice. J Ethnopharmacol 2013;148:826–34.
[24] Oh HA, Kim DE, Choi HJ, Kim NJ, Kim DH. Anti-stress effects of 20*(S)*-protopanaxadiol and 20*(S)*-protopanaxatriol in immobilized mice. Biol Pharm Bull 2015;38:331–5.
[25] Dela Peña IJI, Kim HJ, Botanas CJ, de la Peña JB, Van Le TH, Nguyen MD, Park JH, Cheong JH. The psychopharmacological activities of Vietnamese ginseng in mice: characterization of its psychomotor, sedative-hypnotic, antistress, anxiolytic, and cognitive effects. J Ginseng Res 2017;41:201–8.
[26] Kraeuter AK, Guest PC, Sarnyai Z. The elevated plus maze test for measuring anxiety-like behavior in rodents. Methods Mol Biol 2019;1916:69–74.
[27] Kedia S, Chattarji S. Marble burying as a test of the delayed anxiogenic effects of acute immobilisation stress in mice. J Neurosci Methods 2014;233:150–4.
[28] Lee B, Sur B, Cho SG, Yeom M, Shim I, Lee H, Hahm DH. Ginsenoside Rb1 rescues anxiety-like responses in a rat model of post-traumatic stress disorder. J Nat Med 2016;70:133–44.
[29] Bui BP, Oh Y, Lee H, Cho J. Inhibition of inflammatory mediators and cell migration by 1,2,3,4-tetrahydroquinoline derivatives in LPS-stimulated BV2 microglial cells via suppression of NF-κB and JNK pathway. Int Immunopharmacol 2020;80:106231.
[30] Jeong Y, Bae HJ, Park K, Bae HJ, Yang X, Cho YJ, Jung SY, Jang DS, Ryu JH. 4-Methoxycinnamic acid attenuates schizophrenia-like behaviors induced by MK-801 in mice. J Ethnopharmacol 2022;285:114864.
[31] Pellow S, File SE. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol Biochem Behav 1986;24:525–9.
[32] Domingues M, Casaril AM, Birmann PT, Bampi SR, Lourenço D, Vieira BM, Dapper LH, Lenardão EJ, Sonego M, Collares T, et al. Effects of a selanylimidazopyridine on the acute restraint stress-induced depressive- and anxiety-like behaviors and biological changes in mice. Behav Brain Res 2019;366:96–107.

[33] Miao Z, Wang Y, Sun Z. The relationships between stress, mental disorders, and epigenetic regulation of BDNF. Int J Mol Sci 2020:21.
[34] Bao HY, Zhang J, Yeo SJ, Myung C-S, Kim HM, Kim JM, Park JH, Cho J, Kang JS. Memory enhancing and neuroprotective effects of selected ginsenosides. Arch Pharm Res 2005;28:335.
[35] Kim HJ, Kim P, Shin CY. A comprehensive review of the therapeutic and pharmacological effects of ginseng and ginsenosides in central nervous system. J Ginseng Res 2013;37:8–29.
[36] He B, Chen P, Yang J, Yun Y, Zhang X, Yang R, Shen Z. Neuroprotective effect of 20(*R*)-ginsenoside Rg3 against transient focal cerebral ischemia in rats. Neurosci Lett 2012;526:106–11.
[37] Lee KH, Bahk WM, Lee SJ, Pae CU. Effectiveness and tolerability of Korean Red Ginseng augmentation in major depressive disorder patients with difficult-to-treat in routine practice. Clin Psychopharmacol Neurosci 2020;18:621–6.
[38] Jin Y, Cui R, Zhao L, Fan J, Li B. Mechanisms of *Panax ginseng* action as an antidepressant. Cell Prolif 2019;52:e12696.
[39] Lee S, Rhee DK. Effects of ginseng on stress-related depression, anxiety, and the hypothalamic-pituitary-adrenal axis. J Ginseng Res 2017;41:589–94.
[40] Lee B, Sur B, Cho SG, Yeom M, Shim I, Lee H, Hahm DH. Ginsenoside Rb1 rescues anxiety-like responses in a rat model of post-traumatic stress disorder. J Nat Med 2016;70:133–44.
[41] Cha HY, Park JH, Hong JT, Yoo HS, Song S, Hwang BY, Eun JS, Oh KW. Anxiolytic-like effects of ginsenosides on the elevated plus-maze model in mice. Biol Pharm Bull 2005;28:1621–5.
[42] Carr MN, Bekku N, Yoshimura H. Identification of anxiolytic ingredients in ginseng root using the elevated plus-maze test in mice. Eur J Pharmacol 2006;531:160–5.
[43] Xie X, Yang H, An JJ, Houtz J, Tan JW, Xu H, Liao GY, Xu ZX, Xu B. Activation of anxiogenic circuits instigates resistance to diet-induced obesity via increased energy expenditure. Cell Metab 2019;29:917–31.
[44] Seibenhener ML, Wooten MC. Use of the open field maze to measure locomotor and anxiety-like behavior in mice. J Vis Exp 2015:52434.
[45] Thibaut F. Anxiety disorders: a review of current literature. Dialogues Clin Neurosci 2017;19:87–8.
[46] Xiang M, Jiang Y, Hu Z, Yang Y, Du X, Botchway BO, Fang M. Serotonin receptors 2A and 1A modulate anxiety-like behavior in post-traumatic stress disordered mice. Am J Transl Res 2019;11:2288–303.
[47] Celada P, Puig MV, Casanovas JM, Guillazo G, Artigas F. Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: involvement of serotonin-1A, $GABA_A$, and glutamate receptors. J Neurosci 2001;21:9917–29.
[48] Lladó-Pelfort L, Santana N, Ghisi V, Artigas F, Celada P. 5-HT1A receptor agonists enhance pyramidal cell firing in prefrontal cortex through a preferential action on GABA interneurons. Cereb Cortex 2012;22:1487–97.
[49] Brambilla P, Perez J, Barale F, Schettini G, Soares JC. GABAergic dysfunction in mood disorders. Mol Psychiatry 2003;8:721–37. 15.
[50] Stein L, Belluzzi JD, Wise CD. Benzodiazepines: behavioral and neurochemical mechanisms. Am J Psychiatry 1977;134:665–9.
[51] Zaletel I, Filipović D, Puškaš N. Hippocampal BDNF in physiological conditions and social isolation. Rev Neurosci 2017;28:675–92.
[52] Shimizu E, Hashimoto K, Okamura N, Koike K, Komatsu N, Kumakiri C, Nakazato M, Watanabe H, Shinoda N, Okada S, et al. Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. Biol Psychiatry 2003;54:70–5.
[53] Zhu G, Sun X, Yang Y, Du Y, Lin Y, Xiang J, Zhou N. Reduction of BDNF results in GABAergic neuroplasticity dysfunction and contributes to late-life anxiety disorder. Behav Neurosci 2019;133:212–24.
[54] Martinowich K, Lu B. Interaction between BDNF and serotonin: role in mood disorders. Neuropsychopharmacology 2008;33:73–83.
[55] Madhav TR, Pei Q, Zetterström TS. Serotonergic cells of the rat raphe nuclei express mRNA of tyrosine kinase B (trkB), the high-affinity receptor for brain derived neurotrophic factor (BDNF). Brain Res Mol Brain Res 2001;93:56–63.
[56] Nibuya M, Nestler EJ, Duman RS. Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. J Neurosci 1996;16:2365–72.
[57] Anthony TE, Dee N, Bernard A, Lerchner W, Heintz N, Anderson DJ. Control of stress-induced persistent anxiety by an extra-amygdala septohypothalamic circuit. Cell 2014;156:522–36.