Effect of Ginsenoside Re on Depression- and Anxiety-Like Behaviors and Cognition Memory Deficit Induced by Repeated Immobilization in Rats

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In this study, we assessed the effects of ginsenoside Re (GRe) administration on repeated immobilization stress-induced behavioral alterations using the forced swimming test (FST), the elevated plus maze (EPM), and the active avoidance conditioning test (AAT). Additionally, we examined the effect of GRe on the central adrenergic system by observing changes in neuronal tyrosine hydroxylase (TH) immunoreactivity and brain-derived neurotrophic factor (BDNF) mRNA expression in the rat brain. Male rats received 10, 20, or 50 mg/kg GRe (i.p.) 30 min before daily exposures to repeated immobilization stress (2 h/day) for 10 days. Activation of the hypothalamic–pituitary–adrenal (HPA) axis in response to repeated immobilization was confirmed by measuring serum levels of corticosterone (CORT) and the expression of corticotrophin-releasing factor (CRF) in the hypothalamus. Repeated immobilization stress increased immobility in the FST and reduced open-arm exploration in the EPM test. It also increased the probability of escape failures in the AAT test, indicating a reduced avoidance response. Daily administration of GRe during the repeated immobilization stress period significantly inhibited the stress-induced behavioral deficits in these behavioral tests. Administration of GRe also significantly blocked the increase in TH expression in the locus coeruleus (LC) and the decrease in BDNF mRNA expression in the hippocampus. Taken together, these findings indicate that administration of GRe prior to immobilization stress significantly improved helpless behaviors and cognitive impairment, possibly through modulating the central noradrenergic system in rats. These findings suggest that GRe may be a useful agent for treating complex symptoms of depression, anxiety, and cognitive impairment.

Keywords: Immobilization stress, depression, anxiety, cognition, ginsenoside Re

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Depression is characterized primarily by a chronic depressed mood and is often coupled with sleep disturbances, low self-esteem, guilty feelings, and suicidal tendencies [50]. Thus, this disease is considered a complex disorder, and the mechanisms underlying its pathogenesis remain unclear. The severity of helplessness is frequently combined with symptoms of anxiety and cognitive impairment [10]. In fact, manifestation of pure depression hardly ever occurs without symptoms of anxiety [26]. Although many genetic studies have revealed heritable components in the development of depression, increasingly, it has been appreciated that psychological and social factors, such as stressful life events and chronic stress, cause high vulnerability to depression and induce many long-lasting deleterious effects in the brain [29].

The most important physiological, neuroendocrine, and behavioral abnormality in this disease is hyperactivation of the hypothalamic–pituitary–adrenal (HPA) axis [28]. Active responses to acute stress lead to systemic alterations that intensify the intrinsic ability of an organism to maintain its homeostasis and to minimize the impact of a threat [23]. However, systemic activation against chronic stress can adversely impact brain function [43]. Several animal models using acute restraint stressors have been widely used to investigate the HPA axis response to various stressful stimuli [33, 41]. Previous studies have shown that a maladaptive result from repeated stress led to a high susceptibility to depression, anxiety [22], aggression [35], and cognitive impairment [10].

Animal models with depression- and anxiety-like behaviors and cognitive impairment in response to stressful stimuli are useful in determining the antidepressant, anxiolytic, and anti-amnesic efficacy of candidates in drug screening [10]. Through basic and clinical studies, several antidepressant classes, such as monoamine oxidase inhibitors, selective serotonin reuptake inhibitors (SSRIs), and tricyclic antidepressants (TCAs), have been developed and used clinically for the past several decades. However, most of
these are not very effective against the wide variety of complex depression symptoms, and most are associated with serious side-effects [27]. Recently, more attention has been paid to alternative therapeutics using natural products or medicinal plants for the treatment of stress-related disorders, such as depression, anxiety, and cognitive impairment [12].

Panax ginseng C. A. Mayer and its constituents are frequently used ingredients in Korean traditional herbal medicines for recovery from fatigue, to enhance resistance capabilities against various psychosomatic disorders, to provide various benefits against stress, and to strengthen the immune system [20]. Many studies have been conducted on the biological mechanisms of action of Panax ginseng and its processed product, Korean red ginseng (RG), and it has been reported that these substances possess antidepressant-like activities [42, 52]. Interestingly, several studies have shown that the intensified pharmacological activities of RG are attributable to its component profile of ginsenosides [19]. Although a brief report on an antidepressant activity of protopanaxadiol (PD)-type ginsenoside Rb1 of RG was published [5], it is currently unknown whether treatment with protopanaxatriol (PT)-type ginsenoside Re (GRe), a major active compound isolated from RG, can improve depression- and anxiety-like symptoms and cognitive impairment induced by restraint stress in rats.

The aim of the present study was to investigate the medicinal impacts of GRe on depression- and anxiety-related behaviors and cognitive impairment in rats exposed to repeated immobilization stress using the forced swimming test (FST), the active avoidance conditioning (AAT) test, and the elevated plus maze test (EPM). Moreover, we identified an underlying mechanism, showing how these behavioral effects were associated with the central adrenergic system in rat brain.

**Materials and Methods**

**Animals**

Adult male Sprague–Dawley (SD) rats weighing 240–280 g were obtained from Samtaco Animal Co. (Seoul, Korea). The rats were housed in a limited access rodent facility with up to five rats per polycarbonate cage. The room controls were set to maintain the temperature at 22°C ± 2°C and the relative humidity at 55% ± 15%. Cages were lit by artificial light for 12 h each day. Sterilized drinking water and standard Chow diet were supplied *ad libitum* to each cage during the experiments. The animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996, and were approved by the Kyung Hee University Institutional Animal Care and Use Committee. All animal experiments began at least 7 days after the animals arrived.

**Experimental Groups**

Rats were randomly divided into six groups consisting of seven individuals each as follows: unstressed group daily treated with saline (0.9% NaCl) instead of GRe (CON, n = 7), immobilization-stressed group treated with saline instead of GRe (STR, n = 7), immobilization-stressed and 10 mg/kg GRe-treated group (STR+GRe10, n = 7), immobilization-stressed and 20 mg/kg GRe-treated group (STR+GRe20, n = 7), immobilization-stressed and 50 mg/kg GRe-treated group (STR+GRe50, n = 7), and immobilization-stressed and 10 mg/kg fluoxetine-treated group (STR+FLX, as a positive control, n = 7). According to the protocols of Kim et al. [21], ginsenoside Re from RG was isolated and kindly provided by Korea Ginseng and Tobacco Research Institute (Daejeon, Korea).

Fluoxetine (FLX) was purchased from Sigma-Aldrich Chemical Co. (St. Louise, MO, USA). The rats were intraperitoneally administrated with GRe and FLX 30 min before daily immobilization stress for 10 days, and GRe and FLX were dissolved in 0.9% physiological saline solution before use. All drugs were freshly prepared before every experiment.

The chronic immobilization stress was carried out once daily for 2 h from 10:00 a.m. to 12:00 p.m. and 10 consecutive days in rodent immobilization bags. In brief, rats were forced to be placed in a transparent plastic tube (20 × 7 cm), of which one end is conical shaped and has several 3 mm holes for breathing, and the other end is open. The animals have ample air but were unable to move within the tubes. The following parameters were measured to monitor the effects of the stress: changes of body weight gains (at the beginning step of immobilization stress), and serum CORT levels (after repeated immobilization stress). All rat groups except the CON group received the same immobilization stress. Behavioral testing for depression-like behavior was done 24 h after the end of the stress protocol. The entire experimental schedules of the immobilization stress and behavioral examinations are shown in Fig. 1.

![Fig. 1](image-url)  
**Fig. 1.** Experimental schedules of developing repeated immobilization stress-induced depression- and anxiety-like behaviors and cognitive memory deficit, and the GRe treatment in the rats.

IHC, immunohistochemistry.
Corticosterone (CORT) Analysis
Animals were killed by decapitation one day after behavioral measurements. Blood samples were collected for the determination of serum CORT levels. For this, the unanesthetized rats were rapidly decapitated, and blood was quickly collected via the abdominal aorta. Blood was centrifuged at 4,000 × g for 10 min, and the serum was collected and stored at -20°C until use. The CORT concentration was measured by a competitive enzyme-linked immunosassay (ELISA) using a rabbit polyclonal CORT antibody (OCTESIA Corticosterone kit; Alpco Diagnostics Co., Windham, NH, USA) according to the manufacturer’s protocol. Samples (or standard) and conjugate were added to each well, and the plate was incubated for 1 h at room temperature without blocking. After wells were washed several times with buffers and proper color developed, the optical density was measured at 450 nm using an ELISA reader (MutiRead 400; Authos Co., Vienna, Austria).

**Forced Swimming Test (FST)**
Forced swimming test, a representative behavioral test for depression, is frequently used to evaluate the activities of potential antidepressant drugs in rodent models. Forced immersion of rats in water for an extended period produces a characteristic behavior of immobility. The antidepressant treatments decrease the immobility behavior accompanying with an increase in the escape responses such as climbing and swimming. A transparent Plexiglas cylinder (20 cm diameter × 50 cm height) was filled up to a depth of 30 cm with water at 25°C. At this depth, rats could not touch the bottom of the cylinder with their tails or hind limbs. On day 1, the rats in all groups were trained for 15 min by placing them in the water-filled cylinder. After the test, rats were dried and returned to their home cage. Twenty-four hours later, rats were subjected to 5 min/day for 2 consecutive days of forced swim, and escape behaviors (climbing and swimming) were determined, as previously described [7]. The duration of immobility was scored during the 5 min test period. Climbing was defined as upward-directed movements of the forepaws alone the side of the swim chamber and swimming was considered as movements throughout the swim chamber including climbing into another quadrant. Immobility behavior was calculated as the length of time in which the animal did not show escape responses (e.g., total time of the test minus time spent in climbing and swimming behaviors). The animal’s behavior was continuously recorded throughout the testing session with an overhead video camera. After the test, the rat was removed from the tank, dried with a towel, and placed back in its home cage. The water in the swim tank was changed between rats.

Elevated Plus Maze Test (EPM)
The EPM test is a widely used behavioral test to assess anxiogenic or anxiolytic effects of pharmacological agents [48]. Animals that conduct anxiety-like behaviors usually show reductions both in the number of entries and in the time spent in the open arms, along with an increase in the amount of time spent in the closed arms in the EPM. On day 12, the elevated plus test was conducted. This apparatus consisted of two open arms (50 × 10 cm each), two closed arms (50 × 10 × 20 cm each), and a central platform (10 × 10 cm), arranged in a way such that the two arms of each type were opposite to each other. The maze was made from black Plexiglas and elevated 50 cm above the floor. Exploration of the open arms was encouraged by testing under indirect dim light (2 × 60 W).

At the beginning of each trial, animals were placed at the centre of the maze, facing a closed arm. During a 5 min test period, the following parameters were recorded: (i) number of open arm entries, (ii) number of closed arm entries, (iii) time spend in open arms, and (iv) time spent in closed arms. Entry by an animal into an arm was defined as the condition in which the animal has placed its four paws in that arm. The maze was cleaned with alcohol after each rat had been tested. The behavior in the maze was recorded using a video camera mounted on the ceiling above the center of the maze and relayed to the S-MART program (PanLab, Barcelona, Spain).

**Active Avoidance Conditioning Test (AAT)**
The AAT measures the ability of an animal to avoid an aversive event and provides a way to assess associative learning and memory [14]. Active avoidance conditioning was performed as previously described [14], after finishing the EPM assay. The Gemini Avoidance System (SD Instruments) was used for this experiment. Basically, each rat was individually placed in a two-way shuttle box (23 cm × 50 cm × 23 cm) composed of two stainless steel rods (3 mm diameter, 1 cm apart) and two 28 V DC lights. Electric shocks were transmitted to the grid floor by an isolated shock generator (Behbood Pardaz Co., Iran). First, after a 5 min period of habituation in the shuttle box, the rats were subjected to 50 avoidance trails on variable interval schedule (range = 7.5–22.5 s). Each trial consisted of the warning tone and the stimulus light (conditioning stimulus) for 10 s. If an animal crossed through the archway during the initial 10 s of each trial, the tone and the light were terminated. A conditioned avoidance response was defined as crossing to the opposite chamber within the initial 10 s of each trial. Exactly 24 h after the conditioned avoidance response, rats were again place in the shuttle box. Each trial consisted of the warning tone and the electrical shock (0.5 mA; unconditioning stimulus) for 10 s, presented through the grid floor on the side where the rats were located. If an animal crossed through the archway after the electrical shock was initiated, the tone and the shock were terminated. An escape response was defined as crossing to the opposite chamber within the initial 10 s of each trial. The retention test (escape response) for memory was performed to 20 avoidance trails. If no escape response to the shock occurred within 10 s, the shock and tone-conditioning stimulus were discontinued and an escape failure was recorded.

**Immunohistochemistry of Corticotrophin-Releasing Factor (CRF) and Tyrosine Hydroxylase (TH)**
For immunohistochemical studies, the animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, by intraperitoneal injection) and perfused through the ascending aorta with normal saline (0.9%) followed by 300 ml (per rat) of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, post-fixed overnight, and cryoprotected with 20% sucrose in 0.1 M PBS at 4°C. Coronal sections, 30 mm thick, were cut through the hypothalamus and locus coeruleus (LC) using a cryostat (Leica CM1850; Leica Microsystems Ltd., Nussloch, Germany). The sections were obtained according to the rat atlas of Paxinos and Watson [34].
The sections were immunostained for CRF and TH expression using the avidin–biotin–peroxidase complex (ABC) method. Briefly, the sections were rinsed three times for 5 min each in PBS and then incubated with primary goat anti-CRF antibody (1:2,000 dilution; Santa Cruz Biotechnology Inc., California, CA, USA) and sheep anti-TH antibody (1:2,000 dilution; Chemicon International Inc., Temecula, CA, USA) in PBST (PBS plus 0.3% Triton X-100) for 72 h at 4°C. The sections were washed for 5 min in PBS and then incubated for 120 min at room temperature with biotinylated rabbit anti-goat IgG secondary antibody (for the anti-CRF antibody) and biotinylated goat anti-sheep IgG secondary antibody (for the anti-TH antibody). The secondary antibodies were obtained from Vector Laboratories Co. (Burlingame, CA, USA) and diluted 1:200 in PBST containing 2% normal serum. To visualize immunoreactivity, the sections were incubated for 90 min in ABC reagent (Vectastain Elite ABC kit; Vector Labs. Co., Burlingame, CA, USA), washed three times for 5 min in PBS, and incubated in a solution containing 3,3′-diaminobenzidine (DAB; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 0.01% H₂O₂ for 1 min. Finally, the tissues were washed in PBS, followed by a brief rinse in distilled water, and mounted individually onto slides. The slides were allowed to air dry and were then cover-slipped. Images were captured using the AxioVision 3.0 imaging system (Carl Zeiss, Inc., Oberkochen, Germany) and processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). The sections were viewed at 200× magnification, and the numbers of cells within 100 × 100 µm² grids were counted by observers blinded to the experimental groups. The cells were obtained according to the stereotactic atlas of Paxinos and Watson [34].

Total RNA Preparation and RT-PCR Analysis
The hippocampus from four rats in each group was isolated. After decapitation, the brain was quickly removed and stored at -80°C until use. Total RNA was isolated from the brain samples using TRizol reagent (Invitrogen Co., Carlsbad, CA, USA) and processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). The sections were viewed at 200× magnification, and the numbers of cells within 100 × 100 µm² grids were counted by observers blinded to the experimental groups. The cells were obtained according to the stereotactic atlas of Paxinos and Watson [34]. The cells were counted in three sections for each rat.

**Figure 2.** Effect of GRe administration on body weights (A) and blood levels of corticosterone (B) of the rats under repeated immobilization stress for 10 consecutive days.

- **A:**
  - CON
  - STR
  - STR+GRe10
  - STR+GRe20
  - STR+FLX

- **B:**
  - CON
  - STR
  - STR+GRe10
  - STR+GRe20
  - STR+GRe50
  - STR+FLX

*p<0.05, **p<0.01 vs. CON group; †p<0.05 vs. STR group.

**RESULTS**

**Effect of Ginsenoside Re on Immobilization Stress-Induced Changes of Body Weight and Serum CORT Levels**

Rats exposed to repeated immobilization stress began to lose their body weights at the first day of immobilization (RT-PCR). RT-PCR was performed using a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA, USA). The operating conditions were as follows: for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; for BDNF, 27 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. All primers were designed using published mRNA sequences and primer design software (Primer 3; The Whitehead Institute for Biomedical Research, Cambridge, MA, USA; http://www.genome.wi.mit.edu), offered through the Web site. The following sequences were used: for GAPDH (409 bp), (forward) 5'-ATC CCA TCA CTA TCT TCC AG-3' and (reverse) 5'-CCT GCT TCA CCA CCT TCT TG-3'; for BDNF (153 bp), (forward) 5'-CAG GGG CAT AGA CAA AAG-3' and (reverse) 5'-CTT CCC CTT TTA ATG GTC-3'. The PCR products were separated on 1.2% agarose gels and stained with ethidium bromide, and the density of each band was analyzed using an image-analyzing system (i-Max, CoreBio System Co., Seoul, Korea). The expression levels were compared each other by calculating the relative density of target band, such as BDNF, to that of GAPDH.

**Statistical Analysis**

All measurements were performed by an independent investigator blinded to the experimental conditions. Results in figures are expressed as mean ± standard error of means (SE). Within a behavioral value in the FST, comparisons on day 1 to day 2 used the Student’s t-test, with Levene’s test to make appropriate assumptions of equality of variance. Differences within or between normally distributed data were analyzed by analysis of variance (ANOVA) using SPSS (Version 13.0; SPSS, Inc., Chicago, IL, USA) followed by the appropriate Tukey’s post-hoc test. Statistical significance was set at p < 0.05.
and this initial reduction of body weight was sustained for a while without restoring or even exacerbated in some cases [6, 15]. In the present study, we also examined body weights daily for 10 days to identify whether repeated immobilization stress (STR group) caused body weight loss (differences between daily weights and starting weight) (Fig. 2A). The immobilized rats in the STR group gained less body weight for 10 days than did the normal rats in the CON group. Tukey’s post-hoc test revealed a significant reduction in body weight gain from days 2 to 10 in the STR group, as compared with the CON group. During this period, 50 mg/kg GRe-treated rats showed significant inhibitions of reductions in body weight gains, as compared with the STR group (p<0.05 on days 4, 5, 9, and 10).

Acute immobilization stress induced a large increase in serum CORT level, which gradually decreased as the immobilization stress was repeatedly applied to the rats, probably due to adrenal habituation [36]. In the present study, the serum CORT levels were measured in each group after exposure to repeated immobilization stress for 10 days. The ELISA analysis demonstrated that repeated immobilization stress significantly increased the serum CORT concentration in the rats by 244.33% (p<0.05), as compared with the CON group (Fig. 2B). It indicated that the repeated immobilization stress was sufficiently stressful, despite that the evoked CORT response to repeated immobilization stress was significantly less than the response to single immobilization stress (data not shown). Daily administration of GRe slightly inhibited the immobilization stress-induced increase in serum CORT level as compared with the STR group, but with little statistical significance.

Effect of Ginsenoside Re on Immobilization Stress-Induced Depression-Like Behavior

In a previous experiment, we examined the hypothesis that exposure to immobilization stress exacerbates depressive symptoms associated with the FST, and that the increase in immobility during the first 2 days of the FST (i.e., the ratio of immobility on day 2 to that on day 1) was greater in stressed rats compared with that in unstressed rats. In the present study, normal rats in the control (CON) group did not show significantly increased immobility during the first 2 days of the FST (Fig. 3A; Student’s t-test, p=0.036), which was quantified as an index of immobility on day 2,
normalized to that of day 1 (Fig. 3C). Having confirmed that normal rats in the CON group did not exhibit increased immobility, we analyzed immobility levels in rats in the stress (STR) group that had been subjected to repeated immobilization stress for 10 days. At 24 h following repeated immobilization, the stressed rats in the STR group showed a significant increase in immobility during the first 2 days in the FST (Fig. 3B; Student’s t-test, p<0.05). The normalized immobility duration of the rats in the STR group showed a significant increase in the duration of immobility during the FST compared with the CON group (Fig. 3C; Student’s t-test, p<0.05). This study also examined any ameliorating effect of GRe on immobility in immobilization stress-induced depression-like behavior. Rats in the STR+GRe50 group showed a significant decrease in immobility time during the 5 min in the FTS compared with those in the STR group (p<0.05), indicating that GRe administration effectively decreased depression-like behavior. The results also showed that the reduction of immobility on depression-like behavior in the STR+GRe50 group was almost comparable to that in the STR+FLX group (Fig. 3D).

Similarly, another key behavior manifested as a “climbing behavior” was also investigated [45]. We found the expected increase in climbing behavior during the first 2 days in the FST in rats in the CON group (Fig. 4A; Student’s t-test, p=0.012). Furthermore, rats subjected to repeated immobilization stress showed a significant decrease in climbing behavior (Fig. 4B; Student’s t-test, p<0.05). Using normalized values for climbing behavior, measured as the ratio of time spent climbing on day 2 to that on day 1, the rats subjected to immobilization stress (in the STR group) showed a significant decrease in climbing behavior during the FST, as compared with those in the CON group (Fig. 4C; Student’s t-test, p<0.05). The rats in the STR+GRe50 group showed significant restoration of climbing behavior during the 5 min in the FTS compared with those in the STR group (p<0.05), indicating that the administration of GRe decreased depression-like behavior. It also indicated that the recovery of climbing behavior in the STR+GRe50 group was almost comparable to that in the STR+FLX group (Fig. 4D). However, repeated restraint stress did not induce significant differences in swimming behavior in any group during the FST (data not shown).
Effect of Ginsenoside Re on Immobilization Stress-Induced Anxiety-Like Behavior

The effect of GRe administration on anxiety-like behavior, expressed by a decrease in open-arm exploration in the EPM test, was also investigated (Fig. 5). Post hoc comparisons identified a significant decrease in the percentage of time spent in the open arms of the maze after repeated immobilization stress exposure for 10 days, as compared with that in the CON group (p<0.01). However, the rats in the STR+GRe50 group showed a significant restoration of the percentage of time spent, formerly decreased by repeated immobilization, in the open arm of the maze, as compared with that in the STR group (p<0.05; Fig. 5A). Similarly, post hoc comparisons identified a significant decrease in the numbers of entries in the open arms of the maze after repeated exposure to immobilization stress for 10 days, as compared with those in the CON group (p<0.05). The rats in the STR+GRe50 group also showed a significant restoration in the numbers of entries in the open arm of the maze, as compared with those in the STR group (p<0.05; Fig. 5B). Because any significant differences in the number of closed-arm entries were not observed between groups in the EPM test, it could be suggested that the observed anxiety-like behaviors of the rats with repeated immobilization stress were not attributed to the differences of their locomotion activities (Fig. 5B). The GRe administration without prior input of repeated stress did not elicit an anxiolytic or anxiogenic behavioral activity in this study.

Effect of Ginsenoside Re on Immobilization Stress-Induced Conditioned Avoidance Responses (CAR) and Escape Failures (EF)

To verify the relationship between repeated immobilization stress-induced depression-like behavior and cognitive...
memory impairment and to identify the antidepressant activity of ginsenoside Re, we performed AAT in the immobilization-stressed and ginsenoside Re-treated rats.

The rats in the STR groups showed significant decreases of latencies to enter the opposite chamber for conditioned avoidance responses, as compared with those in the CON group (p<0.05; Fig. 6A). On the other hand, the rats in the STR+GRe20 and STR+GRe50 groups showed significant increases of latencies to enter the opposite chamber for CAR, as compared with those in the STR group (p<0.05).

After CAR trials for 24 h, the effect of GRe administration on retention latency, indicated by the latencies for entering the opposite chamber, was examined by applying electric shock to the grid floor of the shuttle box in the AAT. In the retention, the rats in the STR group exhibited significant increases of latencies to enter the opposite chamber for escape failures, as compared with those in the CON group (p<0.01; Fig. 6B). However, in the STR+GRe50 group, they showed decreased latencies to enter the opposite chamber for EF, as compared with those in the STR group (p<0.05). This study indicated that repeated exposures to immobilization stress severely impaired short-term memory in the rats and that GRe treatment significantly attenuated immobilization stress-induced memory deficit. The rats in the STR group showed a reduction of the acquisition of CAR and an increase in EF. Moreover, the percentage of CAR or EF observed in the STR+GRe50 group was similar to that in the CON group. It also indicated that the recovery of cognitive functioning on stress-related memory impairment in the STR+GRe50 group was almost compatible with that in the STR+FLX group.

**Effect of Ginsenoside Re on Immobilization Stress-Induced CRF- and TH-Like Immunoreactivities**

Following the behavioral tasks, CRF-like immunoreactivity was analyzed in the cell bodies of hypothalamic regions including PVN (Fig. 7). In the rat brains in the STR group, the numbers of CRF immunoreactive fibers in the PVN were increased by 145.60%. Post hoc comparisons revealed that the rats with repeated immobilization stress showed a significant increase in the CRF expression, as compared with those in the CON group (p<0.01). The numbers of CRF-immunoreactive neurons significantly decreased in hypothalamic PVN regions in the STR+GRe50 group (p<0.05), as compared with those in the STR group (Fig. 8). It also indicated that the increased CRF immunoreactivity by repeated immobilization stress was significantly restored by GRe administration, and the numbers of CRF-immunopositive neurons in the STR+GRe50 group was closely associated with those in the CON group.

TH-like immunoreactivity was analyzed in the cell bodies of adrenergic regions, including LC (Fig. 7). In the rat brains in the STR group, the numbers of TH immunoreactive fibers in the LC were increased by 138.76%. Post hoc comparisons revealed that the rats, repeatedly exposed to immobilization stress, showed a significant increase in TH expression, as compared with CON group (p<0.01). The numbers of TH-immunoreactive neurons significantly decreased in central adrenergic regions in the STR+GRe50 group (p<0.05), as compared with those in the STR group (Fig. 8). It also indicated that the increases in the numbers of TH-immunoreactive neurons in the rats with repeated immobilization stress were significantly restored by GRe.

![Fig. 7.](image-url) Representative photographs showing CRF expression in the paraventricular nucleus (PVN) of the hypothalamus and TH expression in the locus coeruleus (LC) of CON-CRF (A), STR-CRF (B), STR+GRe50-CRF (C), CON-TH (D), STR-TH, (E) and STR+GRe50-TH (F) groups. The scale bar indicates 50 µm.
administration, and that the numbers of TH-immunopositive neurons in STR+GRe50 group were closely associated with those in the STR+FLX group.

**Effect of Ginsenoside Re on Immobilization Stress-Induced BDNF mRNA Expression in the Hippocampus**

The effect of GRe administration on the expression level of BDNF mRNA was investigated in the rats with repeated immobilization stress-induced hippocampus alteration using RT-PCR analysis (Fig. 9). The BDNF mRNA expression level was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, a housekeeping gene used as an internal control. The BDNF mRNA expression in the rat hippocampus in the STR group significantly decreased as compared with that in the CON group (p<0.01). The reduced expression of BDNF mRNA in the STR group was significantly restored in the STR+GRe50 group (p<0.05). The restored levels were almost close to those in normal rats in the CON group. The recovery of the expression levels of neuronal markers in the STR+GRe50 group was almost compatible with those in the STR+FLX group.

**DISCUSSION**

Korean red ginseng (RG), one of several processed products of *Panax ginseng*, and its saponin fraction have been shown to possess a variety of pharmacological effects, such as antidepressant properties [20] and antiamnesic activity [17]. Ginsenosides isolated from RG are the major active components, having pharmacological and biological activities [52]. Previous studies have reported that the PD-type ginsenosides, such as ginsenosides Rb1, Rb2, Rb3, Rc, Rd, and Rg3, have antioxidant activities [5], whereas the PT-type ginsenosides such as ginsenosides Re, Rg1, Rg2, and Rf, show efficacy in improving learning and memory functions [49]. Based on these previous studies, we selected ginsenoside Re, a major active component of the PT-type ginsenosides, for the current study.

We examined the dose-dependent activity of GRe (10, 20, or 50 mg/kg), and found that 50 mg/kg was most effective in inhibiting repeated immobilization stress-induced harmful effects, such as depression- and anxiety-like behaviors and memory deficits, using the FST, EPM, and AAT tests. The optimum dose determined in this study was also shown in a previous study [5].

Previous studies on the emotional effects of repeated immobilization stress in rodents have produced controversial results. Several reports have demonstrated that immobilization-
induced stress increased the probability of depression- and anxiety-like behaviors and cognitive impairment, consistent with our results [46]. We observed a gradual decrease in body weight and an increase in serum CORT levels in the animals that experienced immobilization stress. In many studies, it is very well recognized that dysregulation of the HPA axis by repeated immobilization stress or elevated levels of circulating CORT produces hyperactivity of the sympathetic adrenomedullary system, such as CORT, corticosteroid-binding globulin, ACTH, norepinephrine (NE), and epinephrine (EPI) [1, 36]. Administration of GRe significantly restored body weight and serum CORT levels of stressed rats compared with those in the saline-treated control group, suggesting that GRe inhibited HPA axis-associated psychological dysfunction induced by repeated immobilization stress. Previous studies have also shown that the hypothalamic CRF system is involved in the regulation of HPA axis hyperactivity, and depression- and anxiety-like behaviors induced by repeated immobilization stress [9]. Our data suggest that the CRF circuit in the hypothalamus was activated by repeated immobilization stress, leading to the observed anxiety- and depression-like behaviors in the behavioral tests [44]. Furthermore, the expression and secretion of CRF in the PVN of the hypothalamus were significantly increased in the STR group compared with the CON group in the present study. This is consistent with a previous report showing that alterations in CRF underlie depression- and anxiety-like behaviors induced by chronic stress [23]. In our results, the administration of GRe significantly blocked the increase in CRF immunoreactivity in the PVN. These results suggest that CRF modulation in the hypothalamus was involved in the antidepressant and anxiolytic activities of GRe following repeated immobilization stress in rats.

The FST is a valuable and reliable behavioral research model of depression in rodents and is also an important tool to study neurobiological mechanisms involved in antidepressant responses [51]. The observed immobility behavior in the FST is similar to a state of lowered mood or helplessness and depression in humans [13]. In the present study, using a modified version of FST, increased immobility following repeated immobilization stress was clearly observed on day 2 of the test. This result is consistent with previous findings showing that restraint stress or repeated exposure to corticosterone increases immobility duration more on the second day than on the first day in the FST [25]. This observation raises an important question, which has been addressed in earlier studies [18]: what are the differences between immobility on the first day (day 1) and on the second day (day 2), and how does immobility on the first day affect that on the following days in the FST. Because the immobility time on day 2 has been used as a measure of depression, a decrease in the immobility time on day 2 has also been taken to indicate antidepressant activity.

Within the traditional framework of the FST, immobility times on days 1 and 2 have been considered as measures of the response to inescapable stress and depression, respectively [39]. Clearly, in the FST, prolonged immobilization stress for 2 days (days 1 and 2) must be a more aversive experience than a single immobilization stress on day 1, which necessarily affects the subsequent FST behaviors on the second day (day 2) [8]. In the present study, administration of GRe significantly decreased immobility and increased climbing behavior in the FST, but there was no effect on swimming in the FST, confirming an antidepressant-like activity not caused by any change in motor function [40]. Some studies have reported that immobility and climbing behavior in the FST are associated with the central adrenergic system [40]. Thus, our results may suggest that the central adrenergic system was involved in the antidepressant effect of GRe on helpless-like behavior that persisted for 10 days in rats under repeated immobilization stress.

Anxiety is another complex feature of depression, and thus anxiety-like symptoms in chronically stressed animals are not surprising. Many studies have suggested that stressed rats showed decreases in the proportion of time spent in and number of entries into the open arms of the EPM compared with a non-stressed normal control [2, 22]. Although the EPM test is based on conflict and subsequent movement of an animal between an open and illuminated environment and an aversive environment, the test includes two additional anxiety-provoking environmental parameters; height and an open area [11]. In the present study, the administration of GRe prior to each immobilization stress significantly reduced anxiety-like behaviors in the EPM test, as indicated by the increase in the percentage of time in and the number of entries into an open arm. Accordingly, these results suggest that GRe has anxiolytic activity. Many studies also suggest that the anxiety observed in the elevated plus maze test is related to the increase in 5-hydroxytryptamine (5-HT) overflow in the hippocampus [4] and that the repeated exposure to immobilization stress enhances the behavioral measures of anxiety in the test [31]. The repeated immobilization stress thus increased the release and turnover of 5-HT in some areas of the brain, like the hippocampus, which have been implicated in behavioral and physiological responses to these stimuli [4]. Therefore, our results strongly support the hypothesis that administration of GRe inhibits the increase in 5-HT secretion in the hippocampus, attributed to the repeated exposure to immobilization stress. It may play a pivotal role in the neurobiological and behavioral mechanisms by the serotonin system or HPA axis.

An interesting observation showing a correlation between helpless behaviors and cognitive deficits by immobilization stress was reported recently [10]. Some evidence suggests that major depressive disorders are closely associated with a significant deficit in cognitive functioning as well as
recent clinical studies have also shown that long-term administration of antidepressant agents such as TCAs or SSRIs cause a restoration of cognitive function that parallels the mood improvement in depressed patients [16, 37]. It has been demonstrated that the repeated immobilization stress is significantly associated with impairment of cognitive performance, as seen after chronic stress [47]. Our results show that the administration of GRe could ameliorate cognitive deficits in major depression, similar to those induced by repeated immobilization stress in rats. These results also support the potential of GRe as an agent with anti-amnesic efficacy.

Tyrosine hydroxylase (TH) is an enzyme involved in stress-induced activation in the central nervous system and stress-related psychopathological conditions such as depression, anxiety, and cognitive deficit [53]. The ascending noradrenergic neurotransmitter system that originates primarily in the A6 noradrenergic neurons of the LC is a major circuit in the central nervous system involved in the stress response [30]. TH expression in the LC is elevated following repeated exposure to stress [32], possibly due to a long-term adaptive process in anticipation of exposure to subsequent stress. In the present study, TH immunoreactivity in the LC in response to repeated immobilization stress was more marked in the STR group than in the CON group. These results are consistent with previous findings that depression- and anxiety-like behaviors induced by chronic stress are the results of alterations in the central noradrenergic system [38]. Moreover, we demonstrated that the administration of GRe significantly reduced TH-like immunoreactivity in the LC previously activated by repeated immobilization stress. Together, these findings indicate that GRe is capable of attenuating the complex behaviors and neurochemical responses involved in depression and anxiety via modulation of the HPA axis and noradrenergic system.

Furthermore, in a recent experiment, chronic social defeat stress and chronic immobilization stress were associated with a long-lasting down-regulation of BDNF in the brain, which was restored by treatment with an antidepressant [28]. Similarly, cognitive impairment in patients with depression has been tied to abnormal function in the prefrontal cortex and hippocampus [24]. These results suggest that the reduced expression of BDNF in the hippocampus may be related to the pathogenesis of cognitive impairment [28]. In the present study, repeated immobilization stress caused a decrease in the expression of BDNF mRNA in the rat hippocampus as well as learning and memory deficits. However, administration of GRe restored the decreased expression level of BDNF mRNA in the hippocampus in rats under immobilization stress.

In summary, the present study revealed that repeated immobilization stress significantly increased the duration of immobility in the FST and decreased open-arm exploration in the EPM test compared with unstressed normal controls. Moreover, long-lasting altered behaviors including helpless behavior in the FST were correlated with the impaired cognitive function in the AAT in the rats. Furthermore, the administration of GRe significantly reduced depression- and anxiety-like symptoms and cognitive impairment following repeated immobilization stress, possibly through modulation of hypothalamic CRF and noradrenergic system in the central nervous system. Accordingly, GRe may be a useful material in the development of alternative medicines for treating stress-related disorders such as depression, anxiety, and cognitive impairment.

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REFERENCES


