

Anti-Stress Effects of Ginsenoside Rg₃-Standardized Ginseng Extract in Restraint Stressed Animals

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Abstract – We tested whether ginsenosides Rg₃-standardized ginseng extract (RGE) has anti-stress effects in restraint-stressed animals. RGE increased time spent in the open arms and open arm entries in the elevated plus-maze test. In addition, RGE blocked the reduction of center zone distance and stereotypes behaviors in the open-field test. RGE also increased head dips in stressed mice, indicating anxiolytic-like effects. Stress decreased movement distance and duration, burrowing, and rearing frequency but increased face washing and grooming. RGE significantly reversed burrowing and rearing activity in stressed mice. In addition, we measured sleep architecture in restraint stressed rats using EEG recorder. Stress increased rapid eye movement (REM) sleep, but total sleep and non-rapid eye movement (NREM) sleep were not changed. RGE did not affect sleep architecture in stressed rats. These behavioral experiments suggest that RGE has anti-stress effects in restraint-stressed animal models.

Keywords: Anti-stress, Ginsenoside Rg₃-standardized ginseng extract (RGE), Restraint stress, Behaviors, Electroencephalogram (EEG), Sleep

INTRODUCTION

Ginseng, the root of *Panax ginseng* C. A. Meyer (*Araliaceae*) is a traditional medicine in Korea, China, and Japan. Ginseng is an adaptogen, a substance that helps the body resist the adverse influences of a wide range of physical, chemical, and biological factors and helps restore homeostasis irrespective of the direction of altered physiological function (Xiang *et al.*, 2008; Ahmed *et al.*, 2010). The anti-stress activities of ginseng may account for its observed clinical efficacy in stress-related disorders like hypertension, diabetes mellitus, peptic ulcer, and depression and anxiety disorders (Takahashi *et al.*, 1992; Luo *et al.*, 1993; Tachikawa *et al.*, 1999). The hypothalamic-pituitary-adrenal (HPA) axis ensures adaptation and is one of the most important systems for stress responses

(Lian *et al.*, 2005). Ginseng saponins exert various effects on stress and immune systems (Karikura *et al.*, 1991a; Karikura *et al.*, 1991b). Ginseng stabilizes and balances physiology, and may help to maintain normal sleep and wakefulness (Weiss *et al.*, 1998), resulting in clinical use for the treatment of insomnia and stress (Pohorecky and Roberts, 1991; Weiss *et al.*, 1998).

Ginsenosides Rg₃ is a potent ginsenoside with neuroprotective and anti-stress effects (Lee *et al.*, 2006). Ginsenoside Rg₃-standardized ginseng extract (RGE) was obtained by treatment of ginseng extract with 1.0% citric acid at 65°C for 8 hours. We tested whether RGE has an anti-stress effect in restraint-stressed animals. This technique has proven to be a very useful one for the examination of both central and peripheral mechanisms of stress-related disorders, as well as for studying drug effects (Paré and Glavin, 1986).

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MATERIALS AND METHODS

Materials

Six-year-old ginseng was purchased from a herb market in Korea. Ginseng roots were extracted four times with 70% ethanol for 4 h under boiling reflux. The extract was subjected to filtration and concentration. Dried extract was dissolved into distilled water with 1.0% citric acid to transform ginseng saponin to ginsenoside Rg₃. This solution was reacted at 65°C for 8 h. Finally, the ginseng was dried at 90–95°C. Rg₃-standardized ginseng extract contains about 3–4% ginsenoside Rg₃.

Experimental animals

Five-week-old male ICR mice weighing 22–25 g and male Wistar rats (Samtako, Osan, Korea) weighing 250–300 g were used for stress tests. Animals were housed in acrylic cages (45×60×25 cm) with water and food available *ad libitum* under an artificial 12-h light/dark cycle (lights on at 7:00 am) and at constant temperature (22 ± 2°C). To ensure adaptation to the new environment, mice and rats were kept in the departmental holding room for 1 week before testing. This study was performed in accordance with the Chungbuk National University Laboratory Animal Research Center guidelines for the care and use of laboratory animals.

Experiment procedure

Stress procedures were approved and monitored by the ethical committee of Chungbuk National University. The restraint stress was produced by restraining the animals inside an adjustable acrylic hemi-cylindrical plastic tube for 22 hours (for rats, 7.5-cm diameter, 15-cm-long; for mice, 3-cm diameter, 6.5-cm-long). RGE (10, 50 and 100 mg/kg in distilled water) was administered to animals for 5 days once per day. On the 6th day, 1 h after final RGE treatment, animals were stressed for 22 h. For EEG recording, rats were divided into control (non-stress) and stress groups after 7 days post-surgical recovery.

Elevated plus-maze test

The elevated plus-maze apparatus consists of four arms (30×5 cm) elevated 45 cm above the floor, with each arm positioned at 90° relative to the adjacent arms. The two enclosed arms have 30 cm walls, and, to facilitate grip on the open arms, the walls have a raised edge of 0.25 cm. Open and closed arms are connected via a central area (5×5 cm) to form a plus sign. The maze floor is constructed of black Plexiglas, and the walls of the enclosed arms are con-

structed of clear Plexiglas. Four 25-W red fluorescent lights arranged as a cross at 100 cm above the maze are used as the source of illumination, and a video camera is suspended above the maze to record movements for analysis. Mice were randomly assigned (with a slight adjustment for matched body weight) to experimental groups after the stress. Testing was commenced by placing a mouse on the central platform of the maze, facing an open arm. The number of entries into and the time spent on each of the two types of arms were recorded during the 5-min trial (Cha *et al.*, 2004; Kim *et al.*, 2007). An arm entry was defined as all four paws having crossed the dividing line between an arm and the central area. The plus-maze was thoroughly cleaned with 70% methanol after each trial. Mice were randomly allocated to the following groups: control animals (normal saline with 1% CMC, *p.o.*), positive control [diazepam (2.0 mg/kg, *p.o.*)], and RGE (25, 50, 100 mg/kg, *p.o.*).

Hole-board test

The hole-board apparatus (UGO Basile, Italy) consists of gray Perspex panels (40×40 wide, 2.2 cm thick) with 16 equidistant holes, each 3 cm in diameter, on the floor. Photocells below the surface of the hole measure the number of head dips. The board was positioned 15 cm above a table. Mice were treated as above. Each mouse was individually placed on the center of the board facing away from the observer and allowed to freely roam about the apparatus prior to the testing. Diazepam and RGE were administered 30 and 60 min before the test, respectively. The number of head dips on the hole-board was counted for 5 min (Takeda *et al.*, 1998; Wei *et al.*, 2007). After each trial, the floor of the apparatus was wiped with 70% methanol. The test sessions were recorded with a camera mounted vertically above the hole-board.

Open-field test

The open-field test was performed using an ambulatory monitor apparatus (ENV-520 SN 10317). The open-field area was made of acrylic, consisting of four transparent walls and a white floor (30×30×15 cm). The mouse's position in the open-field was classified into five areas: four corner zones and a central zone. RGE and diazepam were administered to the animals 1 h and 30 min before the test, respectively. Each mouse was put in the center of the open field box and tested for 1 h. The time spent and the distance covered in the central zone were recorded. After each individual test session, the floor was cleaned using 70% methanol.

Specific behaviors

After restraint stress, the EthoVision system (Noldus IT b.v., Netherlands) was used to search for six behavior changes: movement, burrowing, face washing, grooming, and rearing activity (Noldus *et al.*, 2001).

Brain surgery and EEG recording

Each rat was implanted with a transmitter (Data Sciences International, TA11CTA-F40, MN, USA) for recording EEG and activity via telemetry. The body of the transmitter was implanted subcutaneously off the midline and posterior to the scapula and was attached to the skin with 3 sutures for stabilization. Leads from the transmitter led subcutaneously to the skull and the bare ends were placed in contact with the dura through holes made in the skull (A: 2.0 [Bregma], L: 1.5; P: 7.0 [Bregma], L: 1.5 contra-lateral). The electrodes were anchored to the skull with screws and dental cement. All surgical procedures were performed stereotaxically under aseptic conditions. Surgical anesthesia was achieved with pentobarbital (50 mg/kg, i.p) and all efforts were made to minimize the suffering of the animals.

Telemetric recording of cortical EEG and activity was conducted using procedures similar to previous reports (Sanford *et al.*, 2006). For the EEG signal, the gain of transmitters was set at $-0.5/+0.5$ volts per/units $\times 2$ and the raw signals generated from the transmitter were in the range of 0.5–20.0 Hz. The signals were processed by a

Data Sciences International analog converter and routed to an AD converter (Eagle PC30, USA) housed in a PC computer. The AD converter digitized the EEG and activity signals at 128 Hz. The digitized data were transferred to the computer and displayed graphically. An on-line fast Fourier transformation (FFT) was performed on the EEG data at 2 sec intervals during data acquisition (256 samples) after a Hanning window treatment. The FFT analysis generated power density values from 0.0 to 20.0 Hz at a resolution of 0.5 Hz. The FFT data were further averaged in the range of 0 to 20 Hz for every 10 sec. The sleep data and FFT results were saved to the hard disk every 10 sec for additional off-line analysis. Movement of the animal in relation to the telemetry receiver generated transistor-transistor logic (TTL) pulses that were collected and counted as a measure of activity. RGE was administered 10 min before the EEG recording. Recording began at 7:00 am for 24 h.

Analysis of sleep architecture

The amount of time in wakefulness, NREM, and REM sleep were determined from the digitized data at 10 sec intervals using sleep analysis software, *SleepSign* 2.1 (KISSEI Comtec Co Ltd., Matsumoto, Japan). Briefly, the software discriminates wakefulness as high-frequency, low-amplitude EEG. NREM was scored based on the presence of spindles interspersed with slow waves in the EEG. EEG power during REM is significantly reduced in lower

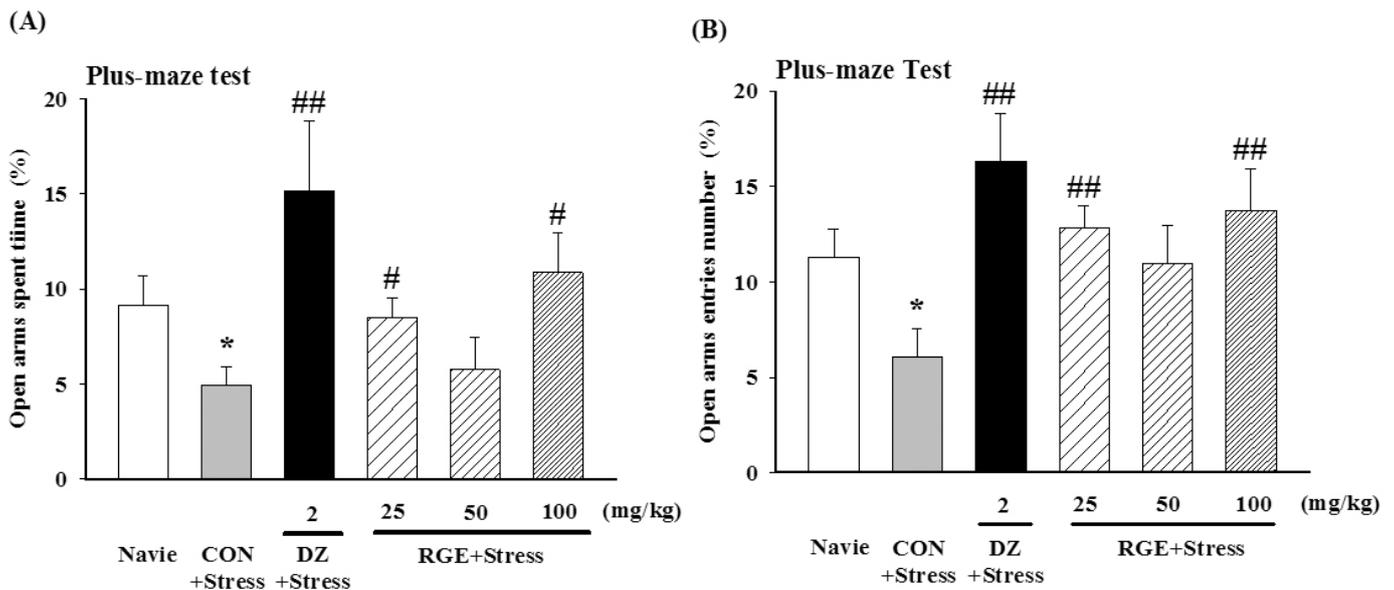


Fig. 1. Anxiolytic-like effects of RGE in the elevated plus-maze test. (A) Time spent in open arms. (B) Open arm entries. Data are expressed as mean \pm S.E.M. * $p < 0.05$, compared to the vehicle-treated control group. # $p < 0.05$, ## $p < 0.01$, significantly different from stress group. More details are in Materials and Methods.

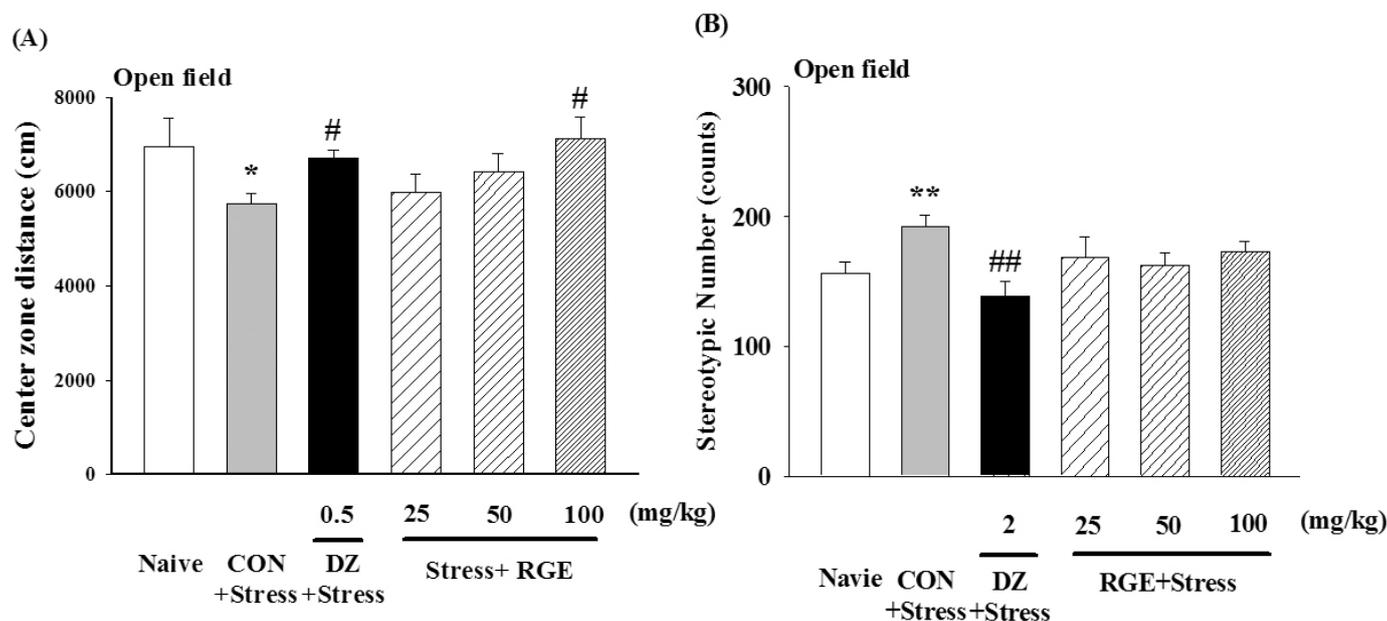


Fig. 2. Anxiolytic-like effects of RGE in the open field test. (A) Center zone distance. (B) Stereotypic Number. Data are expressed as mean \pm S.E.M. * $p < 0.05$, ** $p < 0.01$, compared to the control group. # $p < 0.05$, ## $p < 0.01$, significantly different from stress group. More details are in Materials and Methods.

frequency δ -wave (0.75–4.0 Hz) and increased in the range of θ -wave activity (5.0–9.0 Hz, peak at 7.5 Hz). The time spent (min) in NREM, REM, total sleep time (NREM + REM), and numbers of sleep-wake cycle were processed to obtain 12 h period totals for each rat. We further calculated the time of each recording spent in the sleep-wake state (wake, NREM, REM). The absolute EEG power during wakefulness, NREM, and REM were calculated in 0.5 Hz bins from 0.5 to 20 Hz for the entire 12 h reading of each recording process.

Statistical analysis

All statistical analyses were conducted using *SigmaStat* software. After analysis of variance (ANOVA), post hoc comparisons were conducted using the Tukey test.

RESULTS

Effects of RGE on the elevated plus-maze test

Stress decreased time spent in the open arms and open arm entries in the plus maze test ($p < 0.05$). However, diazepam (2 mg/kg), the positive control, and RGE (25 and 100 mg/kg) blocked these decreases (Fig. 1).

Effects of RGE on the open-field test and hole-board test

Stress increased the incidence of stereotyped behaviors and decreased center zone distance. Diazepam (2 mg/kg)

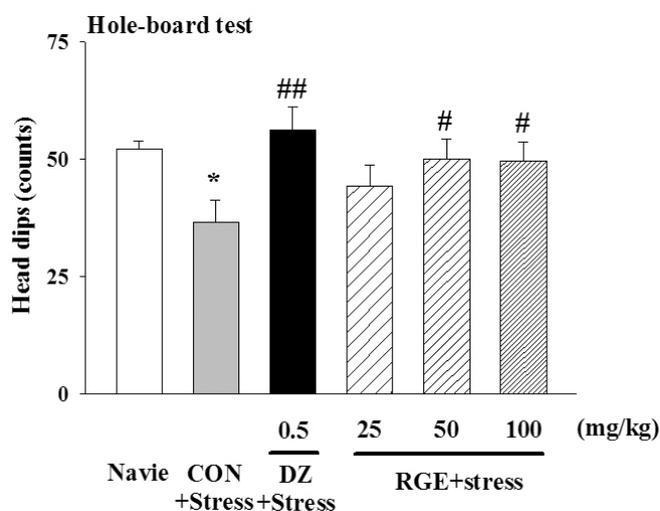


Fig. 3. Anxiolytic-like effects of RGE on hole-board test. Data are expressed as mean \pm S.E.M. * $p < 0.05$, compared to the control group. # $p < 0.05$, ## $p < 0.01$, significantly different from stress group. More details are in Materials and Methods.

significantly decreased stereotyped behaviors ($p < 0.01$) and increased the center zone distance ($p < 0.05$), compared with the naïve group. RGE (100 mg/kg) significantly increased the center zone distance. ($p < 0.05$) (Fig. 2). Stress also decreased head dips on hole-board test ($p < 0.05$). Diazepam and RGE (50 and 100 mg/kg) sig-

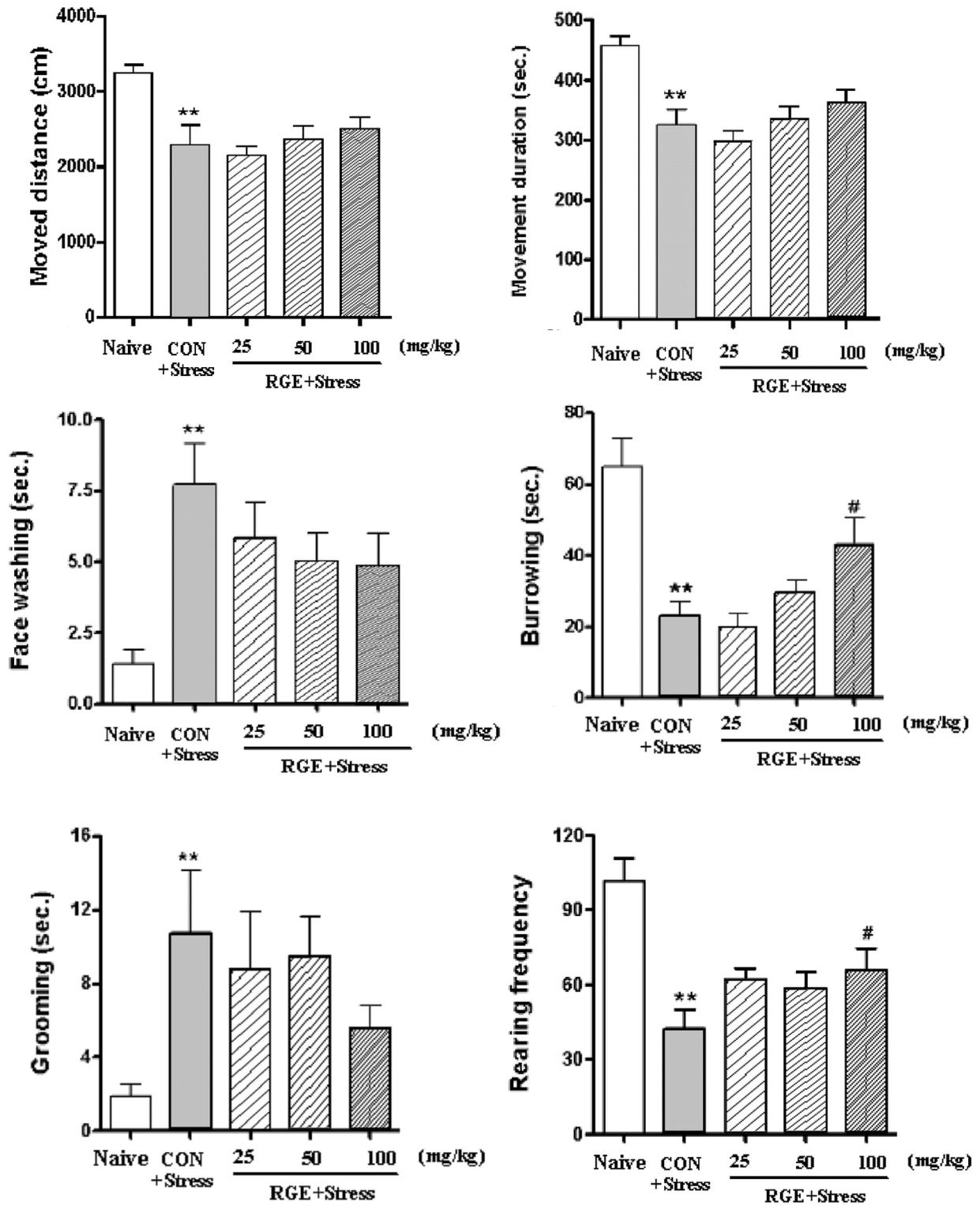


Fig. 4. Effects of RGE on locomotor activity and specific behavior. Data are expressed as mean \pm S.E.M. ** $p < 0.01$, compared to the control group. # $p < 0.05$, significantly different from stress group. More details are in Materials and Methods.

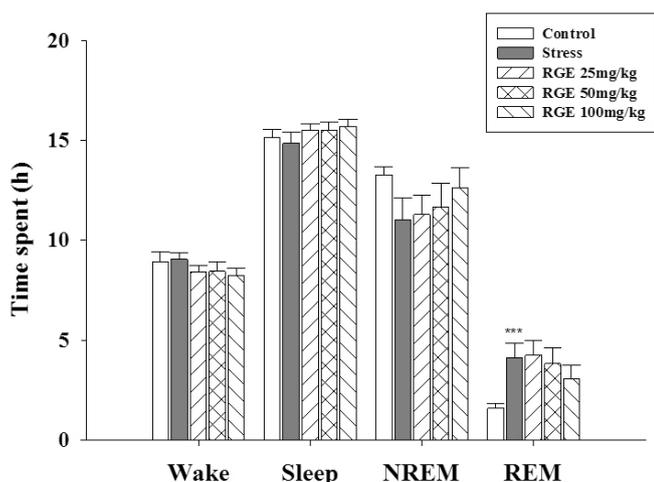


Fig. 5. Effects of RGE on sleep architecture in stressed rats. Data are mean \pm S.E.M of time spent in sleep-wake state (Wake, Sleep (total sleep), NREM sleep, REM sleep) and the number of sleep/wake changes. *** $p < 0.005$, significantly different from control group. More details are in Materials and Methods.

nificantly increased head-dips compared with vehicle control ($p < 0.01$, $p < 0.05$) (Fig. 3).

Effects of RGE on specific behaviors

Stress decreased movement distance and duration ($p < 0.01$) as well as burrowing and rearing, but increased-face-washing and grooming. RGE (100 mg/kg) significantly increased burrowing and rearing activity ($p < 0.05$) (Fig. 4).

Effects of RGE on sleep architecture

Stress increased REM, but did not affect NREM sleep for 24 hours ($p < 0.005$, Fig. 5). RGE (100 mg/kg) did not change wakefulness and total sleep in stressed rats. RGE did not significantly affect in NREM and REM sleep (Fig. 5).

DISCUSSION

Ginseng processing can involve diverse methods such as heating with high pressure or treatment with weak acids. We processed Rg₃-standardized red ginseng using 1.0% citric acid to increase active components and give 3.0-4.0% Rg₃. Others found that Phoenix ginseng is standardized *Panax ginseng* extract with 3.0% Rg₃ (Panwar *et al.*, 2005).

Stress produced anxiety-like behaviors in animals, and RGE and diazepam, an anxiolytic, blocked these effects. Stress decreased time spent in the open arm and number of open arm entries. In stressed animals, the open arm spent time and open arm entries number were decreased,

showing anxiogenic-like effects. Diazepam, a positive control, was increased the open arm spent time and open arm entries number, showing anxiolytic-like effects. Diazepam and RGE both blocked these decreases. Ginseng may affect the GABAergic system in the brain (Han *et al.*, 2009), and RGE may work through a similar mechanism. Here, RGE ameliorated behavioral evidence of anxiety, consistent with ginseng's anti-stress effects, as well as its ability to modulate hypothalamus-pituitary-adrenal axis function and decrease corticosterone levels in blood (Kim *et al.*, 2003; Navarrete *et al.*, 2007). Ginseng components can modulate normal sleep through GABAergic systems (Lee *et al.*, 1990; Attele *et al.*, 1999) and reverse social isolation stress-induced decreases in pentobarbital sleep through GABA_A receptors and neurosteroid function (Matsumoto *et al.*, 1996; Ojima *et al.*, 1997; Ma *et al.*, 2009). RGE may also work through modulating GABA transporters and receptors or neurosteroids.

Glucocorticoid secretion serves both to alert the organism to environmental or physiologic changes and to defend homeostasis under stressful conditions. Increased glucocorticoids can produce physiological and psychological dysfunction, including stress-related disorders (for example, asthma, hypertension, colitis, depression, post-traumatic stress disorder, and dementia). Drugs that suppress the HPA axis may have therapeutic potential for the clinical and endocrine manifestations of depression and stress (Owen *et al.*, 2005). Rg₃ (S) and ginseng extract attenuate stress-induced increases in plasma corticosterone levels in mice (Lee *et al.*, 2006).

Ginseng is used extensively for a wide variety of clinical ailments and to improve general physical and mental well-being (Mittra *et al.*, 1996). Stress increased REM sleep ($p < 0.005$), but RGE unfortunately did affect these changes REM sleep. Our results are consistent with ginseng's effects in ameliorating stress-related sleep disorders, particularly by changing the balance of NREM and REM sleep (Bjorvatn *et al.*, 1998; Shea *et al.*, 2008). These results suggest that RGE may be useful for treatment of stress-related disorders.

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