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Antidepressant-like effect of chlorogenic acid isolated from *Artemisia capillaris* Thunb.

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Artemisia capillaris Thunb. is widely used in the herbal medicine field. This study describes the antidepressant effect of a flavonoid (chlorogenic acid) isolated from the *Artemisia capillaris* Thunb. The expression of the pituitary gland and hypothalamic POMC mRNA or plasma β-endorphin levels were increased by extract of *Artemisia capillaris* Thunb. or its flavoniod administered orally. In addition, antidepressant activity was studied using the tail suspension test (TST), the forced swimming test (FST) and the rotarod test in a chronically restrained immobilization stress group in mice. After restraint stress (2 h/day for 14 days), animals were kept in a cage for 14 days without any further stress, but with drugs. Mice were fed with a diet supplemented for 14 days and during the behavioral test period with chlorogenic acid (30 mg/kg/day). POMC mRNA or the plasma β-endorphin level was increased by the extract of *Artemisia capillaris* Thunb. and its flavoniod. In addition, the immobility time in TST and FST was significantly reduced by chlorogenic acid. In the rotarod test, the riding time remained similar to that of the control group at 15 rpm. Our results suggest that the flavonoid (chlorogenic acid) isolated from *Artemisia capillaris* Thunb. shows a potent antidepressant effect.

Keywords: chlorogenic acid; *Artemisia capillaris* Thunb.; antidepressant; β-endorphin

Introduction

Chronic stress is thought to be a risk factor for psychosomatic psychiatric illnesses, such as anxiety and depression disorders (Ader and Cohen 1993; McEwen and Stellar 1993). Stress alters the homeostasis of many regions of the body, including neural, endocrine, immune and digestive systems. Depression is a disorder characterized by a broad range of symptoms, including altered mood and cognitive functions, and recurrent thoughts of death or suicide. In contrast with the normal experiences of sadness, clinical depression is a chronic disease that can interfere significantly in the individual's life quality. Depression constitutes the second most common chronic condition in clinical practice (Whooley and Simon 2000) and could become the second leading cause of premature death or disability worldwide by the year 2020 (WHO 1999). Approximately two-thirds of anxious or depressed patients respond to the currently available treatments but the magnitude of improvement is still disappointing (Mora et al. 2006).

Excessive stress is related to hyperpiesia, diabetes, gastric ulcers and depression. Reaction against stressful stimuli is necessary to maintain human homeostasis. Endocrine, metabolism, autoimmune and psychological diseases arise when this homeostasis is destroyed. CRH (corticotropin-releasing hormone), arginine vasopressin, peptides, glucocorticoids and catecholamine are related to stress. In particular, POMC is a precursor of β-endorphin composed of active peptides such as ACTH (adrenocorticotropin), melanotropin and α, β and γ-MSH in other brain regions as well as the pituitary gland. These peptides are produced by complex translation of POMC (Krieger et al. 1977).

Although there are many effective antidepressants available today, the current armamentarium of therapy is often inadequate with unsatisfactory results in about one-third of all subjects treated. This necessitates the development of new and more effective antidepressants from traditional medicinal plants whose psychotherapeutic potential has been assessed in a variety of animal models (Zhang 2004).

Artemisia capillaris Thunb. is widely used in the herbal medicine field. It is a common herb, which can dispel wind-heat, subdue hyperactivity of the liver and improve eyesight (Wang et al. 2000; Cha et al. 2005). *Artemisia capillaris* Thunb. has been reported to have hypolipidemic effects in diabetic patients and hyperlipidemic rodents (Pan et al. 1998; Son et al. 2003; Bolkent et al. 2005; Chen and Li 2007). However, despite extensive pharmacological studies, the antidepressant-related effect of *Artemisia capillaris* Thunb. has not been well characterized. Thus, the medicinal importance

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of this plant motivated us to investigate its possible antidepressant effect.

In the present study, the possible antidepressant effect of a flavonoid (chlorogenic acid) isolated from *Artemisia capillaris* Thunb. was examined. Here we demonstrate that chlorogenic acid shows an antidepressant effect in a chronic stress mice model, consisting of restraint for 2 h daily for 14 days.

Materials and methods

These experiments were approved by the University of Hallym Animal Care and Use Committee (registration number: Hallym 2009-05-01). All procedures were conducted in accordance with the 'Guide for Care and Use of Laboratory Animals' published by the National Institutes of Health and the ethical guidelines of the International Association for the Study.

Experimental animals

Male ICR mice (MJ Co., Seoul, Korea) weighing 20–25 g were used for all the experiments. Animals were housed five per cage in a room maintained at $22 \pm 0.5^{\circ}$C with an alternating 12 h light-dark cycle. Food and water were available ad libitum. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were only used once. Experiments were performed during the light phase of the cycle (10:00–17:00).

Oral administration

Oral administration was performed with a gage in a volume of 500 μl/ 25 g bodyweight.

The making of crude extract from Artemisia capillaris *Thunb.*

Artemisia capillaris Thunb. (300 g) was dissolved in 80% ethanol (1500 ml) and extracted by refluxing for 3 h, and then the extract was filtered to obtaining 'A'. This process was repeated again once to obtain 'B' from the residue. 'A' and 'B' were mixed. This mixture was decompressed and dried for use as extract of *Artemisia capillaris* Thunb.

Isolation of total RNA

Three animals from each group were dissected for Northern blot analysis. Total cellular RNA was extracted from dissected pituitary gland and hypothalamus tissues using a rapid guanidine thiocyanate-seperated phenol/chloroform extraction procedure and subsequent precipitation with acidic sodium acetate (Chomczynski and Sacchi 1987). Total cellular RNA in the aqueous phase was precipitated with ice-cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 nm and 280 nm.

Preparation of digoxigenin (DIG)-labeled cRNA probes

The cRNA probe for POMC (Civelli et al. 1982) was synthesized in vitro from linearized expression vectors which contained SP6 or T7 viral promoter. One microgram of linearized plasmid was mixed with RNA labeling mixture that contained ATP, CTP, GTP and DIG-labeled-UTP, transcription buffer, and SP6 or T7 RNA polymerase. After incubation at 37°C for 2 h, the mixture was co-incubated with DNase I (RNase free) at 37°C for 15 min, precipitated in ethanol containing lithium chloride at 70°C for 30 min, and washed with 70% chilled ethanol.

Non-isotopic Northern blot analysis

Isolated RNA samples were dissolved in 40–50 ml water and a 400-fold diluted RNA solution was subjected to spectrophotometric analysis at 260 and 280 nm. Ten micrograms of total RNA and an equal volume of RNA loading buffer (50% glycerol, 1 mM EDTA) were denatured at 65°C for 10 min and subsequently cooled on ice. The denatured RNA samples were electrophoresed on 1% agarose-formaldehyde gels at 60–70 V and transferred to nylon hybond-N hybridization membrane sheets (Amersham, Buckinghamshire, UK). After UV cross-linking, the membranes were prehybridized at 68°C for at least 1 h in a prehybridization buffer (5 × SSC, 50% formamide, 0.02% SDS, 0.1% sodium *N*-lauroyl sarcosine and 2% blocking reagent). The DIG-labeled POMC probe was added to the prehybridization buffer containing 50% formamide. The membranes were incubated overnight at 68°C in a shaking waterbath, and washed twice for 10 min per wash in 2 × SSC and 0.1% SDS at room temperature. Then the membranes were washed twice for 15 min per wash in 0.1 × SSC and 0.1% SDS. After equilibrating the membranes in Buffer I (100 mM maleic acid [pH 7.5] and 150 mM NaCl) for 1 min, the membranes were gently agitated in Buffer II (1% blocking reagent in Buffer I) for 30–60 min. The membranes were hybridized with the diluted anti-DIG-alkaline phosphatase (1:10,000 [75 mU/ml]) in Buffer II for 30 min. After washing the membranes twice for 15 min per wash in 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in Buffer III (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 50 mM $MgCl_2$) for 2 min. Diluted disodium 3-(4-methoxyspiro(1,2-dioxietane-3,2′-[5′-chloro]tricyclo[3.3.1.1^{3,7}]decan)-4-yl)phenyl phosphate (CSPD) (Boehringer Mannheim) (1:100 dilution

in Buffer III) was spread over the surface of the membranes. After incubation of the membranes at 37°C for 15–20 min, the membranes were exposed to Hyperfilm-ECL (Amersham) for detection of the chemiluminescent signal. For rehybridization, blots were washed for 20 min at room temperature in sterilized (Millipore) water. The membranes were washed overnight at 65°C in 50 mM Tris–HCl (pH 8.0), 50% dimethylformamide and 1% SDS to remove the hybridized probe and rehybridized to the DIG-labeled rat cyclophilin cRNA probe, a gene encoding peptidyl-prolyl cis-trans isomerase which is constitutively expressed in most mammalian tissues with the exception of skeletal muscles (Danielson et al. 1988; Takahashi et al. 1989).

Plasma β-endorphin ELISA analysis

The blood sample was collected 90 min after oral administration of the diluted extract (250, 500 and 1000 mg/kg) or chlorogenic acid (10 and 25 mg/kg), and then the serum was obtained by centrifugation (4°C, 1600 × *g*, 15 min). We observed the serum β-endorphin level with ELISA (Phoenix pharmaceutical, INC). The immunoplate in this kit is pre-coated with secondary antibody and nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment will be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in the sample. The biotinylated peptide is able to interact with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution composed of 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen peroxide to produce a blue-colored solution. The enzyme-substrate reaction is stopped by hydrogen chloride (HCl) and the solution turns to yellow.

Restraint stress and drug treatment

The stress treatment procedure has been described previously (Kim and Han 2006). To produce restraint, 8-week-old mice weighing 22–23 g were individually placed head-first into a well-ventilated 50-ml conical tube plugged with a 3-cm-long middle tube and finally with a cap on the 50-ml tube. Mice were not able to move forward or backward in this device. This restraint stress was delivered to animals at set times (2 h) daily from 10 am. Control mice remained in their original cages and were left undisturbed in this home environment. After restraint stress administration, restrained animals were returned to their normal home environments by housing them in pairs. This procedure was repeated for 14 days unless otherwise indicated. After restraint, animals were kept in their normal home cages for an additional 14 days without any further stress, but with drugs. Mice were fed with a diet supplemented for 14 days and during the behavioral test period with chlorogenic acid (Sigma) (30 mg/kg/day). Behavioral tests were performed in the sequence of TST, FST and rotarod tests as described below.

Tail suspension test (TST)

Mice were suspended individually by the tail from a metal rod. The rod was fixed 50 cm above the surface of a table covered with soft cloth in a sound-isolated room. The tip of the tail was fixed using adhesive Scotch tape; the duration of the test was 6 min. The immobility time was determined by an observer, using a stopwatch, who was unaware of the strain (Steru et al. 1985; Bilkei-Gorzo et al. 2002).

Forced swim test (FST)

Mice were placed in a Plexiglas cylinder (height 27 cm, diameter 15 cm) containing water at 24°C and a depth of 14 cm so that they could neither escape nor touch the bottom. Mice were subjected to a 15-min pre-swim. After a 15-min test, the animals were dried quickly with a towel and returned to their home cages. One day later, mice were forced to swim for 6 min. The animals were habituated for the first 1 min and behavior was noted over the next 5 min. Immobility time was defined as the summed time during which animals remained floating with all limbs motionless. This scoring method has been described previously (Porsolt et al. 1977a; Armario et al. 1988, 1991; Kim and Han 2006).

Rotarod test

Mice were evaluated on their motor activity using the rotarod tested at 10 or 15 rpm. The time mice remained on the rotarod was recorded. Maximum time was adjusted to 5 min (Kim and Han 2006).

Drugs

Chlorogenic acid drug was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Figure 1 shows the structure of chlorogenic acid. Extract of *Artemisia capillaris* Thunb. was dissolved in saline. Chlorogenic acid was prepared by the following steps: (A) 1 g of chlorogenic acid was dissolved in 0.5 ml of ethanol plus 0.5 ml of polyethylene glycol 400; (B) separately, 100 mg of sodium carboxymethylcellulose was dissolved in 9 ml of distilled water; (C) finally, Solution (A) and Solution (B) were vigorously mixed. This

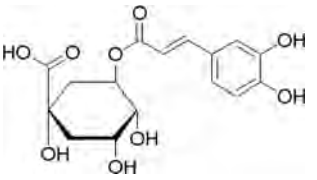

Figure 1. Structure of chlorogenic acid.

solution without chlorogenic acid was used as the vehicle control. All drugs were prepared just before use.

Statistical analysis

ELISA data were presented as the mean ± SEM. The statistical significance of differences between groups was assessed with one-way ANOVA with Bonferroni's post-hoc test using GraphPad Prism version 4.0 for Windows XP (GraphPad Software, San Diego, CA, USA); $P < 0.05$ was considered significant. Behavior test data two-sample comparisons were carried out using the Student *t*-test, and multiple comparisons were made using one-way ANOVA, followed by the Newman-Keuls multiple range test. All data are presented as mean ± SEM, and statistical significance was accepted at the 5% level unless otherwise indicated.

Results

The expression of the pituitary gland and hypothalamic POMC mRNA, and plasma β-endorphin level elicited by extract of Artemisia capillaris *Thunb. administered orally*

Extract of *Artemisia capillaris* Thunb. (250, 500 and 1000 mg/kg) was administered orally to mice, and the hypothalamus was dissected or a blood sample was collected at 90 min after administration of the extract. The control group was administered 0.9% normal saline orally instead of the extract. In the Northern blot assay, we found that POMC mRNA was increased in the extract-treated group compared to the control group by 1000 mg/kg (Figure 2A). With the ELISA method, we found that the plasma β-endorphin level was increased by extract of *Artemisia capillaris* Thunb. (Figure 2B). At 90 min after oral administration, the plasma β-endorphin level was increased significantly in the extract-treated group (250, 500 and 1000 mg/kg) compared to the control group.

Effects of chlorogenic acid on the plasma β-endorphin level in blood.

Chlorogenic acid (Figure 1; 10 and 25 mg/kg) was administered orally in mice, and blood samples were collected after 90 min. With the ELISA method, we found that the plasma β-endorphin level was increased by chlorogenic acid (Figure 3). The plasma β-endorphin level was increased significantly in a dose-dependent manner in the chlorogenic acid-treated group compared to the control group.

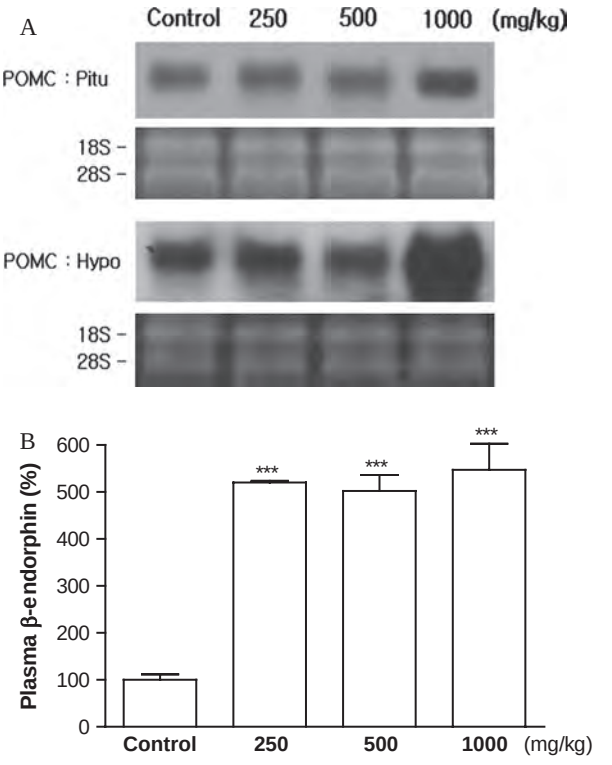

Figure 2. The expression of the pituitary gland or hypothalamic POMC mRNA and plasma β-endorphin level elicited by extract of *Artemisia capillaris* Thunb. administered orally. (A) For Northern blot analysis, the whole mouse pituitary gland and hypothalamus was dissected. Northern blot analysis was performed at 90 min after saline or extract of *Artemisia capillaris* Thunb. (250, 500 and 1000 mg/kg) administered orally. Total constitutively expressed ribosomal RNA (18S and 28S) was used as an internal loading control. Three animals were used for each group. (B) For ELISA, a whole mouse blood sample was collected. ELISA was performed at 90 min after saline or extract of *Artemisia capillaris* Thunb. (250, 500 and 1000 mg/kg) administered orally. The vertical bars indicate the standard error of the mean. The number of animals used for each group was 8–10 (***$P < 0.001$, compared with control group).

Antidepressant-like effects of chlorogenic acid.

After restraint stress (2 h/day for 14 days), animals were kept caged for another 14 days without any further stress, but with drugs. Mice were fed with a diet supplemented for 14 days and during the behavioral test period with chlorogenic acid (30 mg/kg/day). Behavioral tests were performed in the sequence of

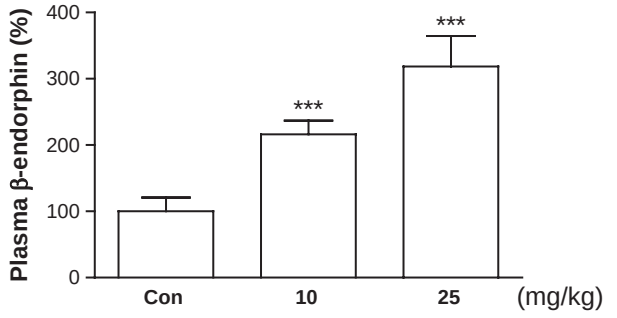

Figure 3. The plasma β-endorphin level elicited by chlorogenic acid administered orally. For ELISA, a whole mouse blood sample was collected. ELISA was performed at 90 min after vehicle control or chlorogenic acid (10 and 25 mg/kg) administered orally. The vertical bars indicate the standard error of the mean. The number of animals used for each group was 8–10 (***$P < 0.001$, compared with control group).

TST, FST and rotarod tests tests as described below (Figure 4A). In the TST test, the immobility time was increased in the chronic restraint stress group compared to the control group. Chlorogenic acid attenuated the total immobilization time to the control level (Figure 4B). In the FST test, the immobility time was increased in the chronic restraint stress group compared to the control group, similar to the result shown with the TST test. Chlorogenic acid attenuated the total immobilization time to the control level (Figure 4C). In the rotarod test, the riding time in the chronic restraint stress group was decreased significantly compared to the control group during at a speed of 15 rpm. Chlorogenic acid returned the riding time to the control level. However, at a speed of 10 rpm, no difference in response was observed in any group (Figure 4D).

Discussion

The opioid system has been implicated in the mechanism of action of some antidepressants. In this regard, it has been proposed that involvement of the opioid system in the antidepressants'mechanism of action may be necessary in order to prove effective in the treatment of severe depression (Schreiber et al. 2002). As mentioned above, stressful system reactivation destroys stress circuit homeostasis, releasing sustained ACTH when chronic stressful stimuli are applied (Sapolsky 2003). In addition, β-endorphin-positive cell bodies in the arcuate nucleus project to the PVN (paraventricular nucleus) (Akil et al. 1984), suggesting that CRH in the PVN of the hypothalamus correlates with β-endorphin-positive neurons in the arcuate nucleus. This suggests that β-endorphin may perform an important physiological role in stress response.

In the present study, we show that POMC mRNA or the plasma β-endorphin level was dose-dependently increased by extract of *Artemisia capillaris* Thunb. and its flavoniod chlorogenic acid. These results suggest that the *Artemisia capillaris* Thunb. and its flavonoid increase β-endorphin, which may perform as an important physiological regulator in response to depression.

The main finding of the present investigation suggests the antidepressant activities of a flavonoid (chlorogenic acid) isolated from the *Artemisia capillaris* Thunb. as manifested in the forced swimming test, tail suspension test and rotarod test models of depression. Chlorogenic acid significantly reduced the immobility period in both TST and FST. In addition, the riding time remained similar to that of the control group in the rotarod test.

The TST and FST are widely used for screening potential antidepressants. Antidepressants reduce the immobility time in both TST and FST. The immobility behavior displayed in rodents when subjected to an unavoidable and inescapable stress has been hypothesized to reflect behavioral despair which in turn may reflect depressive disorders in humans. There is, indeed, a significant correlation between clinical potency and effectiveness of antidepressants in both models (Porsolt et al. 1977b; Steru et al. 1985; Cryan et al. 2002). The rotarod test is a classic model for observing central nervous system actions providing information about psychomotor performance, anxiety and depression. To establish a practical animal model for chronic stress studies, in the present study we examined the usefulness of the rationale that the psychiatric traits anxiety and depression-related behavior can be used as stress assessment parameters. After restraint stress (2 h/day for 14 days), animals were kept caged for another 14 days of period without any further stress, but with drugs. Mice were fed with a diet supplemented for 14 days and during the behavioral test period with chlorogenic acid (30 mg/kg/day).

Pretreatment with chlorogenic acid exhibited a significant effect on immobility time in TST and FST. So, the escape-directed behaviors with minimal immobile posture showed by the chlorogenic acid-treated rats may be due to its attenuating effect in endogenous depression. The underlying principle measuring the lack of active coping behavior is identical in the TST and FST, but their variability in response to certain antidepressants indicates potentially different substrates and neurochemical pathways mediating performance in these tests. These issues may underlie the observed behavioral differences (Bai et al. 2001). Furthermore, one of the most important differences between these two models is the response to drugs in both tests and the apparent increased sensitivity of the

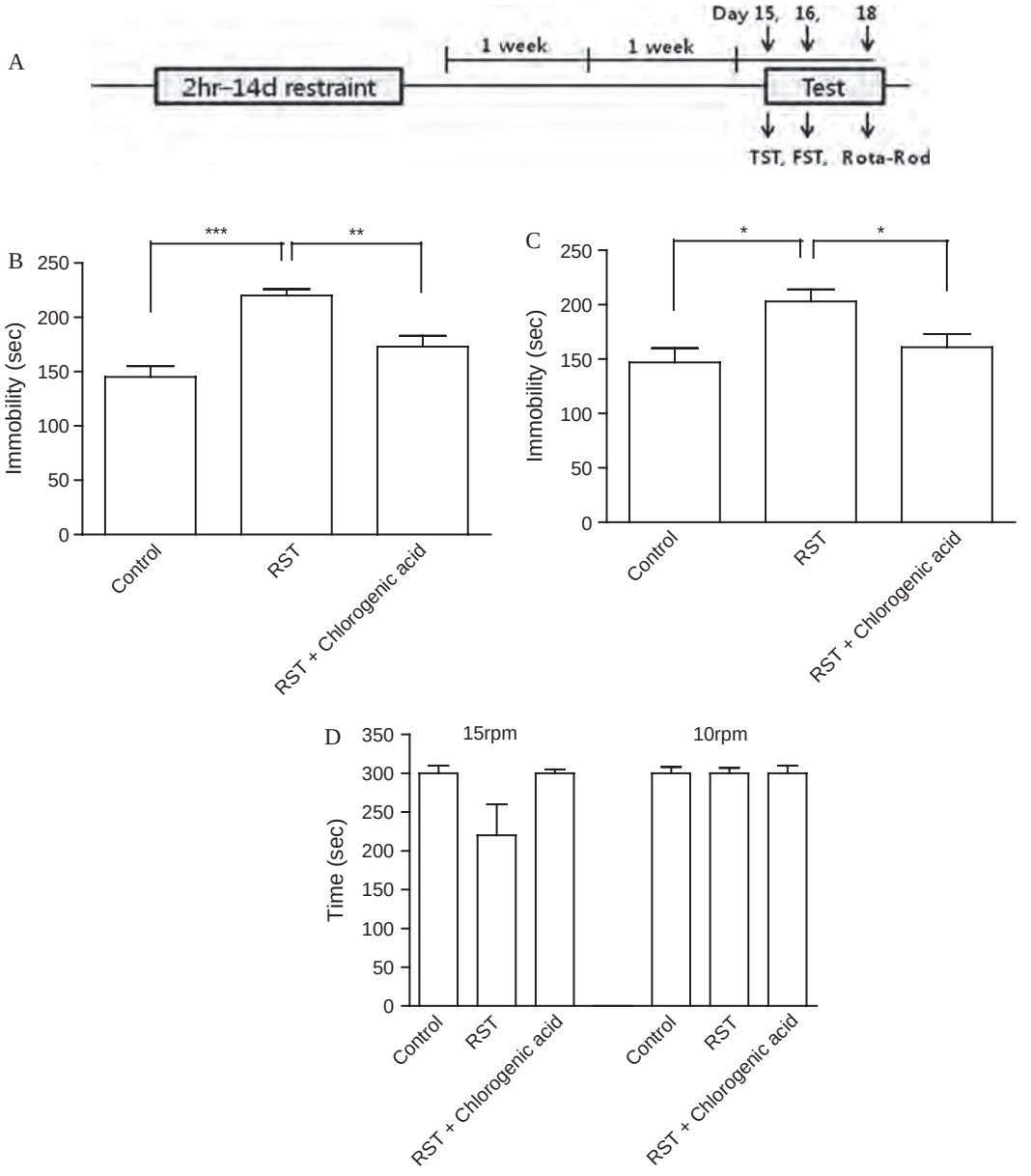

Figure 4. Effect of chlorogenic acid on immobility time in the TST, FST and rotarod test. Mice were fed with a diet supplemented for 14 days and during the behavioral test period with chlorogenic acid (Sigma) (30 mg/kg/day) (A). Behavioral analyses were performed in the following sequence: (1) tail suspension test (TST) 2 weeks after the final stress loading (on day 15) (B); (2) forced swimming test (FST) 2 days after (on day 16) (C); and (3) the rotarod test on day 18 (D). The vertical bars indicate the standard error of the mean. The number of animals used for each group was 8–10 ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with control group).

TST. The mouse FST has not traditionally been viewed as a consistently sensitive model for detecting selective serotonin reuptake inhibitor activity, whereas these antidepressants are generally reported as active in the TST (Cryan et al. 2005). Moreover, the TST was proposed to have a greater pharmacological sensitivity as compared with the FST (Thierry et al. 1986; Cryan et al. 2005). Remarkably, TST detects the anti-immobility effects of a wide array of antidepressants, including tricyclic antidepressants (TCA), selective serotonin reuptake inhibitors (SSRI), monoamine oxidase inhibitors (MAOI), electro-convulsive shock (ECS) and even atypical antidepressants. Thus, the activity of chlorogenic acid could involve one of the mechanisms of the established agents as described above.

In summary, our results suggest that *Artemisia capillaris* Thunb. and a flavonoid (chlorogenic acid) isolated from the *Artemisia capillaris* Thunb. exert an

antidepressant effect in experimental animal depression models. Thus, *Artemisia capillaris* Thunb. chlorogenic acid can be developed as a useful remedy for treatment of depression.

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