Antidepressant-like activity of Chaihu-Shugan-San aqueous extract in rats and its possible mechanism

Sue Wang[1,2], Suiyu Hu[1,2], Chunhu Zhang[1,2], Juan Qiu[3], Yunhui Li[1,2]

[1]Department of Integrative Medicine, Institute of Integrative Medicine, Xiangya Hospital, Central South University, [2]Department of Integrative Medicine, Key Unit of State Administration of Traditional Chinese Medicine of the People's Republic of China, Changsha, Hunan, [3]Department of Nuclear Medicine, Xiangya 3rd Hospital, Central South University, Changsha, Hunan, China

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ABSTRACT

Background: Chaihu-Shugan-San (CHSGS), a traditional Chinese medicinal formula, is commonly used for the treatment of depression in China. However, the molecular mechanism underlying its antidepressant action is unknown. **Objective:** The objective of this study is to evaluate the antidepressant-like effects of CHSGS and further explore the possible molecular mechanism implicated in its actions. **Materials and Methods:** The rats were randomly divided into four groups: The normal control group, the model control group, the CHSGS group and the fluoxetine control group. The antidepressant-like effects of CHSGS aqueous extract were assessed in rats exposed to chronic mild stress (CMS) using the open-field test and sucrose water consumption test, its underlying mechanism of anti-depression was explored by determining the effect of CHSGS on the extracellular signal-regulated kinase (ERK) and phospho-ERK (P-ERK) in the hippocampus using western blot. The aqueous extract of CHSGS at a dose of standard (5.9 g/kg·d) was administered intragastrically for 14 days during the CMS model while the fluoxetine control group was given at the same time using fluoxetine hydrochloride (1.8 mg/kg·d). **Results:** The stressed rats demonstrated decreased locomotor activity in open field test and reduction in sucrose consumption and decreased levels of P-ERK1/2 and the ratio of P-ERK1/2 to total ERK1/2 in the hippocampus. CHSGS alleviated the depressive-like behaviors and increased levels of P-ERK1/2 and the ratio of P-ERK1/2 to total ERK1/2 in stressed rats as well as fluoxetine. **Conclusion:** In summary, these results suggest that CHSGS aqueous extract possesses an antidepressant-like activity in CMS induced depression model rats, which might be mediated, at least in part, by reversing the stress-induced disruption of ERK activity.

Key words: Antidepressant-like activity, Chaihu-Shugan-San, extracellular signal-regulated kinase, hippocampus

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INTRODUCTION

Depression is a common disorder associated with high rates of chronicity, relapse and recurrence, psychosocial and physical impairment and a high suicide rate; It is a devastating illness and a major contributor to the global disease burden.[1,2] In spite of the introduction of tricyclic antidepressants, monoamine oxidase inhibitor, selective serotonin reuptake inhibitor, noradrenergic reuptake inhibitor and serotonin and noradrenaline reuptake inhibitor, rather poor tolerability of currently used antidepressants and late onset of their therapeutic effects further increase the risk of unsuccessful treatment.[3-5]

Address for correspondence:
Dr. Sue Wang, Institute of Integrative Medicine, Xiangya Hospital, Central South University, No. 87, Xiangya Road, Changsha, Hunan 410 008, China. E-mail: wangsue0909@yahoo.com.cn

Therefore, the search for new antidepressants with no or lower side-effects continues and some of the herbal medicines may be successful alternatives in the treatment of depression.[6] Chaihu-Shugan-San (CHSGS), a traditional chinese formula, is composed of seven herbs (Radix Bupleuri, Aurantii nobilis Pericarpium, Szechwan Lovage rhizome, Nutgrass Galingale rhizome, Fructus aurantii, Paeonia and Glycyrrhiza uralensis) and is widely used clinically to relieve a wide variety of symptoms caused by liver-qi stagnation.[7] In previous studies, we have determined the main constituents of CHSGS formula, involving albiflorin, ferulic acid, paeoniflorin, liquiritin, naringin, hesperidin, merazin hydrate, neohesperidin, isoliquiritigenin, glycyrrhizic acid, a-cyperone and 18-bata-glycyrrhizic acid. And we further investigated and found that ferulic acid, naringin, merazin hydrate and neohesperidin may be its antidepressant active ingredients.[8,9] It has been demonstrated efficacy and

safety not only in animal models, but also in clinical trials for treatment of depression;[10-12] however, the molecular correlates underlying its antidepressant action merit further investigation.

The etiology and pathogenesis of depression is quite complex. Chronic stress has been reported to be the essential factor for this disease, stressful life-styles and events often precede the onset of clinical depression and stress-related neuronal plasticity mechanisms are proposed to be involved in the pathogenesis of affective disorders.[13,14] More recent studies have demonstrated that the extracellular signal-regulated kinase (ERK) signal pathway regulating neuronal plasticity participated in stress response and correlated with the stress-induced depressive-like behaviors;[15] in addition, brain-derived neurotrophic factor can ameliorate depression through activating the ERK pathway,[16] and fluoxetine treatment can exert mood-elevating effect and reverse the stress-induced decrease of phospho-ERK2 (P-ERK2) in the hippocampus and prefrontal cortex.[17] These data suggest that the ERK signal pathway may be the molecular mechanisms of antidepressant action and pathophysiology of depression. Yet it is notable that there is no study to the date to investigate the effect of CHSGS on the stress-induced alterations in ERK pathway.

In the current study, we aim to evaluate the antidepressant-like effects of CHSGS by using the open-field test and the sucrose water consumption test; In addition, the expression of P-ERK1/2 and total ERK1/2 in the hippocampus were assayed by western blot to explore the possible association between its antidepressant-like effect and ERK1/2 activity.

MATERIALS AND METHODS

Plant materials and preparation of extracts

The CHSGS prescription and dosage: According to The People's Republic of China Pharmacopeia 1st volume conventional dosage: *Radix Bupleuri* 9 g, *Aurantii nobilis pericarpium* 9 g, *Szechwan Lovage rhizome* 9 g, *Nutgrass Galingale rhizome* 9 g, *Fructus aurantii* 9 g, *Paeonia* 15 g, *Glycyrrhiza uralensis* 5 g. The composition and the amount of each component in percentage of whole medicine are shown in Table 1. Component herbs of CHSGS were purchased from Hunan Herbal Materials Company (Changsha, China) and authenticated by Professor P. Lei, Xiangya Hospital, Central South University based on their microscopic and macroscopic characteristics. The quality of these crude drugs is controlled by the Pharmacopeia and a voucher specimen of each plant was prepared and deposited in the herbarium of the Department of Pharmacognosy of Xiangya Hospital, Central South University. After being decocted, concentrated and finally dried in vacuo, the CHSGS formula was made into a dried powder; the yield of the CHSGS aqueous extract was ca. 12.5% (w/w). The dried extract was stored at −20°C until its use and re-suspended in distilled water for its administration to experimental animals.

Table 1: Composition of Chaihu-Shugan-San

Medicinal plant	Amount (g)
Radix Bupleuri	9
Aurantii nobilis Pericarpium	9
Szechwan Lovage rhizome	9
Nutgrass Galingale rhizome	9
Fructus aurantii	9
Paeonia	15
Glycyrrhiza uralensis	5

Animals

All experiments and procedures were approved by the Central South University Institutional Animal Care and Use Committee.

40 male Sprague-Dawley (SD) rats from Experimental Animals Centre of Central South University, Changsha, Hunan and weighing 180-220 g at the beginning of the experiment were used. The animals were housed 5 per cage under controlled conditions of 12/12 h light-dark cycle (8:00-20:00), background noise (40 ± 10 dB) and temperature (20 ± 3°C) with food and water available *ad libitum*. The animals were acclimatized to the laboratory for at least 7 days before the stress procedure. The rats were randomly assigned to four groups by ballot: A group for CHSGS, a model group, a normal group and a group for fluoxetine (n = 10 per group).

The statistical analysis showed that there was no significant difference in body weight among different groups, which had comparability. In the CMS model, 30 SD rats were housed singly.

Stress procedure

The chronic stress model was induced by chronic unpredictable mild stress (CMS). After 7 days habituation, the rats in stressed groups were housed singly and then subjected to the CMS procedure once daily for 4 consecutive weeks. Each week, the stress regimen followed the method of Willner *et al*.[18,19] with some modifications and consisted of the following stressors: 24-h food deprivation; 24-h water deprivation; 24-h reversed light/dark; 5-min cold swim at 4°C; 5-min thermal stimulus in 45°C oven; electric foot shock (10 mA electricity was given every other minute, which last 10 s/time for 30 times); intermittent white noise (85 db); 24-h soiled cage; 24-h 45° cage tilt and 1-min tail clip. The same stressor was not applied successively

so that rats could not anticipate the occurring of stress. Immediately after the conclusion of each stress session, the animals were returned to their home cages and maintained in standard conditions until the next stress session. Normal control animals were housed in group of 5 per cage without disturbing except for necessary procedure such as weighting or cage cleaning. They had free access to food and water except for a 24-h period of food and water deprivation before the sucrose consumption test.

Drug administration

The dried CHSGS extract and fluoxetine (purity >98%; Eli Lilly and Co., Indianapolis, IN, USA) were dissolved in distilled water before administration, respectively. All dosages were expressed as milligrams per kilogram body weight of the respective drugs. The normal group and the model group received only distilled water. Food, but not water, was withdrawn from the animal 1-h prior to drug administration.

The CHSGS extract 5.9 g/kg/day, fluoxetine 1.8 mg/kg/day and distilled water were intragastrically administered to rats of each group respectively at a volume of 4.5 ml/kg/day once daily for consecutive 2 weeks from the 3rd week. The dosage of medicines administered to the rats equaled to that of a 70 kg- adult. In the following studies, open-field test or sucrose consumption test in rats was conducted 1-h after the last drug treatment, respectively.

Open-field test

The open-field test was performed according to the method described by Papp *et al*.[20] with modifications and was carried out before stress, 2 weeks after stress and 4 weeks after stress. The open-field apparatus was a four-sided 100 cm × 100 cm × 40 cm wooden enclosure, with floor painted white and divided into 25 equal squares by black lines and side walls painted black. Tests were conducted in a darkened, quiet room lit by a 60-W light bulb suspended above the center of the open field. Each rat was gently placed onto the middle square of the open-field apparatus facing away from an observer and allowed to explore freely. The number of crossing sector lines with all four paws and the number of rearings on its hind limbs were recorded during a test period of 3 min. After each animal was tested, the test apparatus was cleaned with a detergent to eliminate odors for the next test.

Sucrose consumption test

The procedure was performed as described previously.[21] Rats were trained to consume 1% (w/v) sucrose solution before the experiment. They were exposed to two bottles of 1% sucrose solutions in a 24-h period and exposed to one bottle of 1% sucrose solution and one bottle of drinking water for next 24-h period. Formal test was carried out before stress, 2 weeks after stress and 4 weeks after stress. Animals were food and water-deprived for 24-h and they were exposed to both the test solution (1% sucrose and drinking water) for the next 1-h period. Sucrose consumption was measured by reweighing pre-weighed bottles of test solution. Bottles were counterbalanced across left and right sides of the cages throughout the experiment, varying for each test and each animal.

Western blotting

Immediately after the sucrose consumption test, all rats were decapitated and the brains were rapidly removed on ice. The hippocampus were dissected bilaterally according to the atlas of Paxinos and Watson.[22] Tissues were homogenized in 20 volumes of buffer (pH 7.4, containing 20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA Ethylenediamine tetraacetic acid disodium, 1 mM EGTA Ethyleneglycol bis (2-aminoethyl) tetraacetic acid, 1% Triton X-100, 2.5 mM sodium orthovanadate, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). Protein content of lysates was determined using bicinchoninic acid (Pierce Biotechnology, Rockford, IL, USA). Lysates were mixed with 5 × sodium dodecyl sulfate (SDS) to prepare for certain concentration of sample solutions. Thereafter proteins were separated by 10% SDS-polyacrylamide gels and were blotted onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA) by electrophoretic transfer. Blots were incubated in blocking buffer (5% nonfat dry milk powder in tris-buffered saline containing 0.1% Tween-20, [TBST]) for 1 h at room temperature and washed 3 times with TBST for 10 min each. Blots were then incubated overnight at 4°C with 1:1000 diluted primary antibodies including mouse anti-β-actin, rabbit anti-ERK1/2 and mouse anti-P-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then washed 3 times for 10 min each in TBST. Blots were incubated with appropriate Horseradish peroxidase -labeled goat anti-mouse or goat anti-rabbit secondary antibody IgG (Santa Cruz) for 2 h at room temperature, washed 3 times for 10 min each in TBST, treated with ECL reagents (Amersham) and exposed to film. Band intensity was quantified by Glyko BandScan software (Glyko Inc., Novato, CA, USA). The relative level of each signal protein was calculated as the ratio of total gray of each signal protein/β-actin.

Statistical analysis

All data were presented as the mean ± standard error of mean (SEM) and analyzed by SPSS v. 12.0 (SPSS Inc., Chicago, IL, USA) statistical package. Multiple group comparisons were performed using one-way analysis of variance followed by least significant difference test for comparison of means. The P values lower than 0.05 ($P < 0.05$) were considered significant.

RESULTS

Effects on open-field activity scores in open-field test

Open-field activity was measured 3 times during the experimental period [Figure 1]. In the open-field test, there were no significant differences in the number of crossings and rearings among all groups before stress ($F(3,36) = 0.108$, $P > 0.05$ and $F(3,36) = 0.031$, $P > 0.05$, respectively). Rats in model group showed a significant decrease in the number of crossings and rearings after 2-week stress compared with the normal control group ($P < 0.01$), which continued to the 4th week when the experiment was closed ($P < 0.01$). In both of drug treated groups, the number of crossings and rearings decreased significantly after 2-week exposure to stressor ($P < 0.01$) and show a significant increase compared with the model group after 2-week treatment ($P < 0.01$) and did not show significant differences in these behavioral changes between CHSGS group and fluoxetine group after 2-week treatment ($P > 0.05$).

Effects on sucrose consumption

Sucrose consumption was measured 3 times during the experimental period [Figure 2]. Before stress, there were no significant differences among the four groups ($F(3,36) = 0.797$, $P > 0.05$). After 2-week stress, the sucrose consumption in stressed rats was significantly lower than that in the normal control group ($P < 0.01$). After 2-week exposure to stressor the sucrose consumption in the CHSGS and fluoxetine groups was significantly lower than that in the normal control group ($P < 0.01$) and show a significant increase compared with the model group after 2-week treatment ($P < 0.01$).

The levels of P-ERK1/2 and total ERK1/2 in the hippocampus

The signals of β-actin, P-ERK1/2 and ERK1/2 were detected in the hippocampus [Figure 3]. There were significant differences among groups in the hippocampus in the levels of p-ERK1/2 ($F(3,16) = 7.352$, $P < 0.01$ and $F(3,16) = 3.432$, $P < 0.05$, respectively) [Figure 4a]; CMS rats showed a significant decrease in the levels of P-ERK1/2 in the hippocampus as compared with normal group ($P < 0.01$ and $P < 0.05$, respectively); administration of CHSGS (5.9 g/kg/body wt.) for 14 days increased levels of p-ERK1/2 in the hippocampus as compared with the model control group ($P < 0.01$ and $P < 0.05$, respectively); the positive control, fluoxetine (1.8 mg/kg/body wt.) also increased levels of P-ERK1/2 significantly ($P < 0.01$ and $P < 0.05$, respectively) and there were no significant differences in the levels of P-ERK1/2 between CHSGS group and Fluoxetine group ($P > 0.05$, either). However, for the levels of ERK1/2, no significant difference was observed among groups in the hippocampus ($F(3,16) = 1.390$, $P > 0.05$ and $F(3,16) = 0.850$, $P > 0.05$, respectively) [Figure 4b].

There were significant differences among groups in the hippocampus in the ratio of P-ERK1/ERK1 and

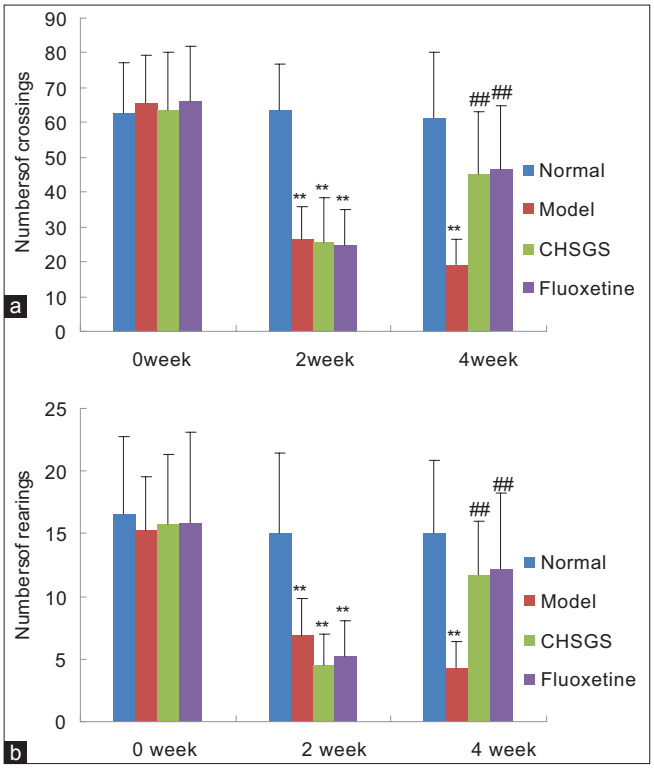

Figure 1: Open-field activity of rats. Rats were administrated with Chaihu-Shugan-San or fluoxetine for 2 weeks after 2-week exposure to the stressor. The open-fi eld test was carried out before stress (0 week), 2 weeks after stress (2 week) and 4 weeks after stress (4 week). (a) Number of crossings during the 3 min session. (b) Number of rearings during the 3 min session. Values are the means ± standard error of mean ($n = 10$). $^{**}P < 0.01$, as compared with the normal control group; $^{\#\#}P < 0.01$, as compared with the model group

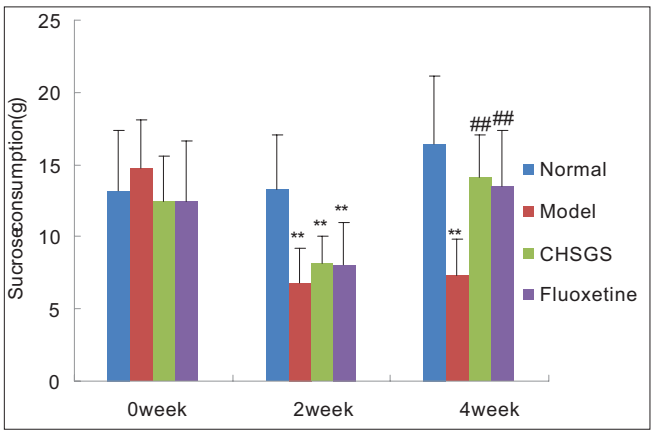

Figure 2: Sucrose consumption of rats. Rats were administrated with Chaihu-Shugan-San or fl uoxetine for 2 weeks after 2-week exposure to the stressor. The sucrose consumption test was carried out before stress (0 week), 2 weeks after stress (2 week) and 4 weeks after stress (4 week). Results are expressed as the means ± standard error of mean ($n = 10$). $^{**}P < 0.01$, as compared with the normal control group; $^{\#\#}P < 0.01$, as compared with the model group

P-ERK2/ERK2 ($F(3,16) = 3.327$, $P < 0.05$ and $F(3,16) = 3.572$, $P < 0.05$, respectively) [Figure 4c]; the CMS rats had a marked decrease in the ratio of P-ERK1/ERK1 and P-ERK2/ERK2 ($P < 0.05$, either); CHSGS or fluoxetine had a reversing effect on the stress-induced decrease in the ratio of P-ERK1/ERK1 and P-ERK2/ERK2 (CHSGS: $P < 0.05$ and $P < 0.01$, respectively; fluoxetine: $P < 0.05$, either) and there were no significant differences in the ratio of P-ERK1/ERK1 and P-ERK2/ERK2 between CHSGS group and Fluoxetine group ($P > 0.05$, either).

DISCUSSION AND CONCLUSION

Stress has been recognized as an important factor involved in the development of depression;[14] the rat CMS model, an ethologically relevant animal model of depression, has been used widely to study behaviors associated with depression, mechanisms of antidepressant actions and several biochemical changes.[23-25] The open-field test has been used widely to assess emotionality and locomotor performance.[20,26] Furthermore, the use of sucrose consumption is valid in relation to the two core symptoms of major depression, i.e., anhedonia and depressed mood.[21,26] In this study, depression-like model states were prepared in rats subjected to a combination of housing singly and a series of daily CMS analogs to "stressful life-styles and events" implicated in the etiology of depression.[27,28] After 14 days of exposure to chronic stress from which they cannot escape, the stressed rats exhibited behavioral deficits including decreased body weight gain, decreased locomotor activity in open-field and reduction in sucrose consumption. At the same time, the present study indicated that CHSGS or fluoxetine reversed almost all behavioral alterations observed in the model. Our results are in concordance with data from other studies, which demonstrate that chronic CHSGS treatment reverses chronic mild stress (CMS) - induced behavioral disorders and confirmed previous studies.[10-12] It thus appears that there is significant efficacy for CHSGS in treatment of depression.

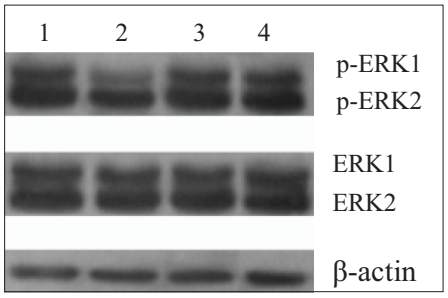

Figure 3: Representative western blots of phospho-extracellular signal-regulated kinase (P-ERK1/2), total ERK1/2 and their corresponding normalized control β-actin in hippocampus. (1) Normal group; (2) model group; (3) Chaihu-Shugan-San group; (4) fluoxetine group

The neuronal mechanisms that turn stress signals into behavioral disorders are far from understood. Studies thus far, however, have suggested that the mitogen-activated protein kinase (MAPK) signaling transduction pathways contribute to various stress-induced synaptic plasticity and may be the mechanism of antidepressant action and the pathophysiology of depression.[29,30] ERK1/2 is the most studied members of MAPK family and the ERK1/2 pathway is the major convergence point in all signal pathways, When activated, the phosphorylation state of ERK1/2, P-ERK1/2 can translocate into nucleus carrying extracellular stimuli primarily regulating cellular growth and differentiation and neuronal plasticity.[31] ERK1 and ERK2 are prominently found in the hippocampus, which is one of the brain regions most likely to be implicate stress response and depression.[32,33] In the recent studies, researchers have indicated that ERK1/2 signal transduction pathway may be not only related to stress-induced behavior disorders but involved in the molecular mechanism of antidepressant action.[15,30] The signal conduction molecule, ERK1/2, plays an important role in stress-induced activity of neurons in brain; and P-ERK1/2, its active form, has been used as a new marker for the activity of neurons in functional morphology study.[15,30,31] It was found in this research that, after the inducing of chronic stress, levels of P-ERK1/2 in model rats' hippocampus evidently reduced, especially P-ERK1; And the ratio of the P-ERK1/2 to

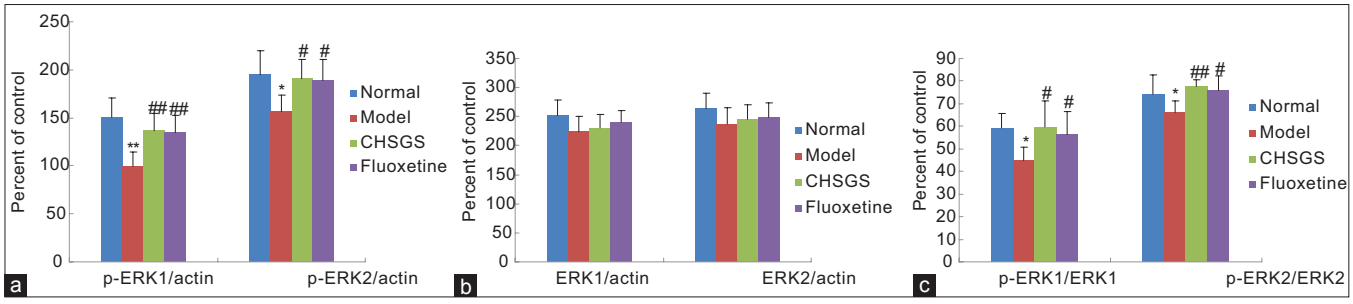

Figure 4: The levels of phosphor-extracellular signal-regulated kinase (P-ERK1/2) (a), ERK1/2 (b) and the ratio of P-ERK1/2 to total ERK1/2 (c) in hippocampus. All data were expressed as mean ± standard error of mean (n = 5/group). $^{**}P < 0.01$ compared with normal group. $^{*}P < 0.05$ compared with normal group. $^{\#\#}P < 0.01$ compared with model group. $^{\#}P < 0.05$ compared with model group

total ERK1/2 was also decreased in the hippocampus. However, no statistical difference between the rats exposed to 28-day-CMS and the normal group was observed in ERK1/2 levels in the hippocampus. CHSGS or fluoxetine reversed the stress-induced disruption of P-ERK1/2 and increased the ratio of the P-ERK1/2 to total ERK1/2, which is indicated by the increased levels of P-ERK1/2 and the increased ratio of the P-ERK1/2 to total ERK1/2 in the hippocampus in CHSGS group and fluoxetine group compared with model group. Our results are in accordance with previous reports, which indicate decreased P-ERK1/2 in depressed human beings and in depressed rats.[34,35] Although recent documents indicate that lithium, valproate, fluoxetine and fluvoxamine, some medications largely used for the treatment of bipolar disorder illness can exert mood-elevating effect through activating the ERK pathway;[17,36,37] the effect exerted by CHSGS on the ERK signal system in brain has not been documented and the present study demonstrated for the first time that CHSGS reversed the stress-induced decrease of the P-ERK1/2 levels and increased the ratio of P-ERK1/2 to total ERK1/2 in the hippocampus and the statistical analysis showed that the effect of CHSGS was similar to fluoxetine.

In conclusion, the present study demonstrated that CMS decreased P-ERK1/2 levels and the ratio of P-ERK1/2 to total ERK1/2 in the hippocampus and induced depressive-like behaviors and CHSGS alleviated the depressive-like behaviors and increased levels of P-ERK1/2 and the ratio of P-ERK1/2 to total ERK1/2. Basing on the results, the mechanism that CHSGS exerts antidepressant actions might be mediated by reversing the stress-induced disruption of ERK activity.

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