The American Journal of Chinese Medicine, Vol. 37, No. 2, 261–272

Institute for Advanced Research in Asian Science and Medicine

Sini Tang Prevents Depression-Like Behavior in Rats Exposed to Chronic Unpredictable Stress

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Abstract: Sini Tang, a Chinese traditional prescription containing three herbs, has been widely used for *Yang*-deficiency. Recent clinical studies have shown that Sini Tang could treat and improve depression symptoms, but the mechanisms underlying the antidepressant effect of Sini Tang remains unknown. In rats with chronic unpredictable stress (CUS), we examined the effects of Sini Tang on sucrose preference and open field exploratory behavior. The levels of corticosterone level in plasma and corticotropin- releasing hormone (CRH) mRNA expression in hypothalamus were also measured by enzyme-linked immunosorbent assays (ELISA) and real-time reverse transcription PCR (RT-PCR), respectively. Rats subjected to CUS exhibited decreases in sucrose preference and ambulation in the open field test. These were all attenuated by Sini Tang in a dose-dependent manner. Biochemically, Sini Tang also reversed CUS-induced increases in corticosterone in plasma and CRH mRNA in the hypothalamus. The behavioral effects of the Sini Tang were correlated to the biochemical actions. These results suggest that Sini Tang produces an antidepressant-like effect, which appears to involve CRH in the brain.

Keywords: Depression; Chronic Unpredictable Stress; Corticotropin-releasing Hormone; Corticosterone; Radix aconiti lateralis preparata, Rhizoma zingiberis recens; Leguminosae.

Introduction

Major depression is a mental disorder characterized by a pervasive low mood and loss of interest or pleasure in usual activities (Chen *et al*., 2008b). Today, Depression is the leading cause of disability and the 4th leading contributor to the global burden of disease. Current

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treatment is dominated by antidepressants that exert their therapeutic effects through an interaction with serotonin (Apparsundaram *et al*., 2008). However, serotonin is just one of many factors that may play a role in the depression. New research points to other biological contributors to depression, including inflammation, elevated stress hormones, immune system suppression, or abnormal activity in certain parts of the brain (Wong and Licinio, 2001). Meanwhile, even though newer types of antidepressants are better tolerated and are safer in overdose than the older tricyclic compounds, they still produce troublesome side-effects (Cassano and Fava, 2004). Thus, better antidepressants with greater efficacy and fewer side-effects are needed.

In traditional Chinese medicine (TCM), traditional Chinese prescriptions and formulae have been used by TCM practitioners in China for thousands of years and are based on principles aimed to produce either synergism or antagonism; this medical approach has played an important role in the prevention and treatment of many diseases in Chinese history (Leung, 2007; Wang *et al*., 2007; Jordan and Tu, 2008). Traditional Chinese prescriptions have been commonly recognized as safe and effective in the treatment of various depressive disorders in China (Kang *et al*., 2005; Chen *et al*., 2008a). Sini Tang, a famous traditional Chinese medical formula, has been widely used to treat *Yang*-deficiency. Recent clinical studies have shown that Sini Tang could treat and improve depression symptoms (Qin *et al*., 2005; Sun *et al.,* 1998). However, the mechanisms underlying antidepressant effect of Sini Tang remains unknown.

To address this issue, we examined the effects of the Sini Tang in rats exposed to chronic unpredictable stress (CUS) as this stress paradigm produces behavioral deficits thought to model aspects of depression (Katz, 1982; Rygula *et al*., 2005; Willner, 2005). The antidepressant-like potential of Sini Tang was then evaluated by using behavioral alterations caused by CUS. Corticotropin-releasing hormone (CRH) is the major regulator of the hypothalamus-pituitary- adrenal (HPA) axis and it modulates important brain functions such as anxiety, learning, food intake, and locomotion. CRH stimulates production of corticosterone and is involved in anxiety and mood disorders, suggesting that CRH and corticosterone may play significant roles in the pathophysiology of depression (Kvetnansky *et al*., 2006; Louvart *et al*., 2006). Therefore, the effect of Sini Tang on the level of corticosterone and CRH mRNA expression in rat hypothalamus was also investigated.

Materials and Methods

Animals

Forty eight male Sprague-Dawley rats (Beijing Weitong Lihua Research Center for Experimental Animals), weighing between 200 and 220 g, were used in the experiments. They were randomly divided into 4 groups: (1) the normal control, (2) CUS, (3) normal dose Sini Tang (according to equal clinical dose), and (4) high dose Sini Tang. Rats were kept on a 12:12 hours in the light: dark cycle in individual home cages with food and water available*ad libitum* except as described in stress. All experiments conformed to the guidelines of China legislations on the ethical use and care of laboratory animals. All efforts were made to minimize animal suffering and the number of animals needed to produce reliable data.

Drugs and Drug Treatment

Sini Tang was composed of the following dried raw materials: Radix aconiti lateralis preparata, Rhizoma zingiberis recens and Leguminosae. These 3 herbs, purchased from medicinal Materials Company of Beijing Tongrentang, were extracted by boiling water for 2 times. Then the 3 fractions were filtered and centrifuged at 3,000 rpm for 40 min; the upper layer of water extract was concentrated properly and dried *in vacuo* (70°C) and made into dry powders with volatile constituents. The doses were expressed in terms of the dry weight of the decoction extract per unit body weight of the experimental animals (g/kg).

Three groups of rats (group 2, 3 and 4) were exposed to CUS, given oral saline (1 ml/kg), and Sini Tang (2.5 and 10 g/kg), respectively. Drug was suspended in distilled water and administered 1 hour before the stress exposure for 21 days.

CUS Procedure

The chronic stress procedure was modified from procedures used by Heine *et al.* (2004). Briefly, rats were exposed to different stressors daily for 21 days as follows. Day 1: cold immobilization for 1 hour at 4°C, forced swim for 30 min at 25°C; Day 2: immobilization for 1 hour, crowding for 24 hours; Day 3: forced cold swim stress for 5 min at 10°C, isolation for 24 hours; Day 4: immobilization for 1 hour, vibration for 1 hour; Day 5: forced swim stress for 30 min at 25°C, cold immobilization for 1 hour at 4°C; Day 6: forced cold swim stress for 5 min at 10°C, crowding for 24 hours; Day 7: vibration for 1 hour, isolation for 24 hours. This schedule was repeated twice for a total of 21 days. To exclude effects of handling of the stressed rats, control rats were handled twice daily. Prior to the study, certain criteria were set for excluding animal on weight loss, or the possible occurrence of wounds.

Sucrose Preference Tests

Sucrose preference tests were used to operationally define anhedonia. Specifically, anhedonia was defined as a reduction in sucrose intake and sucrose preference relative to the intake and preference of the control group. A sucrose preference test consisted of first removing the food and water from each rat's cage for a period of 20 hours. Water and 1% sucrose were then placed on the cages in preweighed glass bottles, and animals were allowed to consume the fluids freely for a period of 1 hour. Two baseline preference tests were performed, separated by at least 5 days, and the results were averaged. A preference test was also conducted following the 21 day CUS period. Sucrose and water consumption (ml) was measured and the sucrose preference was calculated as the sucrose preference (%) = sucrose consumption/(sucrose consumption + water consumption).

Open Field Exploratory Behavior Test

Open field test was used to study the exploratory and anxiety behavior of rats. The open field apparatus consisted of a square arena 60 × 60 cm with a 40 cm high wall. The entire

apparatus was painted black except for 6 mm white lines that divided the floor into 16 equal size squares. The apparatus was illuminated with a low intensity diffuse light (45 W) situated 45 cm above the floor level. Entire room, except the open field was kept dark during the experiment. Each animal was placed in the central square and observed for 5 min and the following behaviors were recorded. Ambulation — the number of grid lines it crossed with all four paws; rearing — by counting the number of times the animal stood on its hind limbs; grooming-number of times the animal made these responses viz. grooming of the face, licking/cleaning and scratching the various parts of the body, defecation — the number of fecal boli excreted during the period and immobility period. Between tests, the apparatus was cleaned with 5% alcohol.

Plasma Hormone Analysis

Immediately after the last stress regimen, animals were sacrificed by decapitation and blood was collected in EDTA coated tubes kept in ice and centrifuged at 1000 × g for 20 min at 4°C. Plasma was separated and aliquots were stored at −70°C for corticosterone estimation. The rat hypothalamus was also carefully separated to measure CRH mRNA expression. Corticosterone level in plasma was determined by enzyme-linked immunosorbent assays (ELISA) as previously described (Kim *et al*., 2007; Tian *et al*., 2008).

mRNA Level of CRH

CRH mRNA in hypothalamus was measured by real-time reverse transcription PCR (RT-PCR) as previously described (Wang *et al*., 2008). The total RNA from different experimental conditions was obtained by TRIzol method. The concentration of RNA was determined by an absorbance at 260 nm and RNA was reverse transcribed to cDNA using the Taqman? Reverse Transcription Reagents (Applied Biosystems). Reverse transcription was performed at 20°C 5 min, 42°C 60 min, 70°C 5 min (Perkin-Elmer GeneAmp 9600, Foster City, CA). cDNA was analyzed immediately or stored at −20°C. Real-time quantitative PCR analyses for COX and GAPDH were performed in 96-well plates using the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems). PCR were performed with the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol, using the following oligonucleotide primers: CRH-forward 5′-TGT CCG AAA GGG CGA TTA-3′, and reverse 5′-GCG GTG CTG AAG CTA TGT AC-3′ (184 bp); β-actin-forward 5′-ACG TTG ACA TCC GTA AAG AC-3′ and reverse 5′-GGA CTC ATC GTA CTC CTG CT-3′ (239 bp). The basic protocol for real-time PCR was an initial incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 1 min and finally cooling to 4°C. All samples were run in triplicate, the relative expression values were normalized with β-actin value. Plasmids containing cDNA was used as standard in quantifying the PCR results. The interest cDNA was amplified by RT-PCR using the same premiers as for real-time RT-PCR. The PCR products were cloned into pGEM-T easy vector (Invitrogen) and confirmed by sequencing. The purified recombinant plasmid DNA was quantified by UV spectrophotometer and then

serially diluted in double-distilled water as standard for numerical quantification. The standard curve prepared for CRH and β-actin was used as housekeeping gene. The PCR products were sequenced to verify the analytical specificity. Melting curve was analyzed after PCR amplification.

Data Analysis

Results were expressed as mean $\pm$ S.E.M. Data were analyzed by one-way ANOVA followed by Dunnet's multiple comparisons using GraphPad Prism 4.0, statistics software. In all the tests, the criterion for statistical significance was $p < 0.05$ (Wang *et al*., 2005).

Results

Sucrose Preference Tests

CUS induced a decrease in sucrose preference, relative to control conditions, which is indicative of operationally defined anhedonia. Figure 1 displays the preference for sucrose in the control, CUS and Sini Tang groups at baseline and following 21 days of CUS. Prior to conducting the study, an ANOVA yielded no significant effects, these four groups did not differ in their baseline sucrose preference ($p > 0.05$). Following 21 days of CUS, the preference for sucrose was significantly reduced in the CUS group relative to the control group ($p < 0.01$). Treatment with Sini Tang 2.5 g/kg or 10 g/kg augmented the decrease in sucrose preference ($p < 0.05$), however did not completely reverse the sucrose preference change induced by CUS.

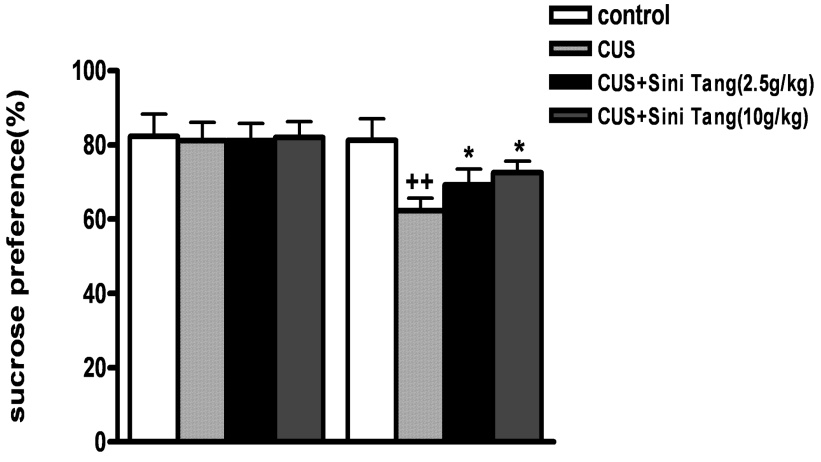


Figure 1. Sucrose preference in the control, chronic unpredictable stress (CUS), and Sini Tang groups following 21 days of CUS were shown. Treatment with Sini Tang significantly augmented the decrease in sucrose preference compared to CUS group ($p < 0.05$). $^{+}$ and * indicates statistical significance in comparison to the control and CUS groups respectively and denotes $^{+,*}p < 0.05$; $^{++,**}p < 0.01$.

Table 1. Effect of Saini Tang on Open Field Exploratory Test in Rats After 21 Days of CUS

Groups	Ambulation Central	Rearing	Grooming	Immobility Period (sec)
Control	9.31 ± 0.46	25.53 ± 3.97	5.32 ± 0.98	59.31 ± 5.83
CUS	4.57 ± 0.73**	8.54 ± 1.77**	13.44 ± 1.36**	224.36 ± 19.17**
CUS +Sini Tang (2.5g/kg)	5.46 ± 0.58	$16.32 \pm 1.24^{\Delta}$	$8.92 \pm 0.57^{\Delta}$	$102.54 \pm 10.22^{\Delta\Delta}$
CUS +Sini Tang (10g/kg)	6.63 ± 0.53	$22.58 \pm 1.43^{\Delta\Delta}$	$6.78 \pm 1.34^{\Delta\Delta}$	$71.23 \pm 8.60^{\Delta\Delta}$

Data are expressed as mean $\pm$ S.E.M. $^{+}$ and * indicates statistical significance in comparison to control and CUS groups respectively and denotes $^{+},{}^{*}p < 0.05$; $^{++},{}^{**}p < 0.01$.

Open Field Exploratory Behavior Test

CUS rats exhibited anxious behavior as evidenced by decreased ambulation, rearing, increased grooming and immobility period ($p < 0.01$) in comparison to control rats. Treatment with Sini Tang significantly reversed the stress induced behavioral alteration in a dose-dependent manner as observed by increased ambulation, rearing and decreased immobility period as compared to the CUS group. In the open field exploration the CUS rats evinced significant decrease in the central activity in comparison to control rats. Though Sini Tang treated rats exhibited an increase in central action it was not significant (Table 1).

Plasma Hormone Analysis

Exposure to CUS led to significant elevation in plasma corticosterone level compared with control ($p < 0.01$). Further, treatment with Sini Tang (2.5 and 10 g/kg) significantly countered CUS induced elevation in levels of plasma corticosterone in a dose-dependent manner ($p < 0.05$ and $p < 0.01$, respectively). The results were graphically represented in Fig. 2.

CRH Gene Expressions

Real-time quantitative PCR was used to measure the mRNA level of CRH in rat hypothalamus. The standard curve was drawn for CRH and β-actin gene (for example, CRH in Figs. 3A and 3B). Melting curve analysis confirmed that there was no primer dimer in the PCR products (Fig. 3C). For each primer set, non-specific amplification was seen after agarose gel electrophoresis and ethidium bromide staining (Fig.3D). The relative CRH gene expression levels were shown as ratios to the mean levels in control rats (Fig. 4). CRH mRNA expression was upregulated in CUS group compared with the control group ($p < 0.01$), while it was significantly suppressed in the Sini Tang group in a dose-dependent manner ($p < 0.05$ and $p < 0.01$, respectively).

Discussions

The chronic mild stress model of depression as described by Willner *et al.* (1987) is accepted as a valuable method for inducing experimental depression in rats. In the model, various stressors are applied in an unpredictable order, simulating conditions in the natural environment.

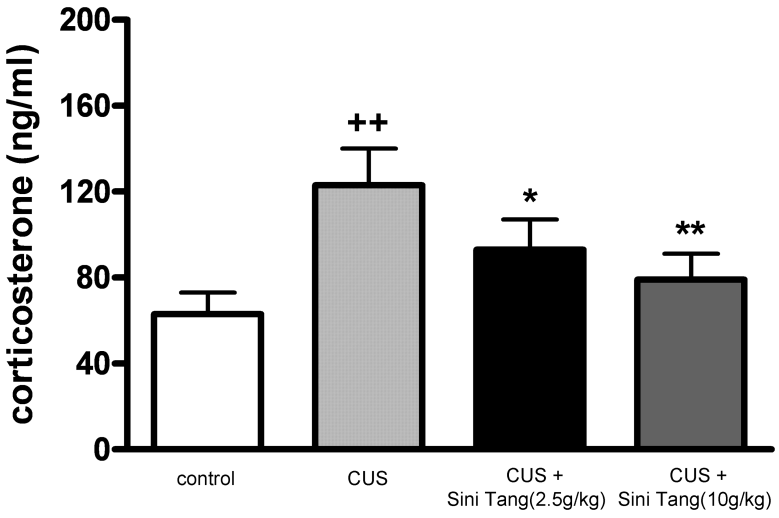
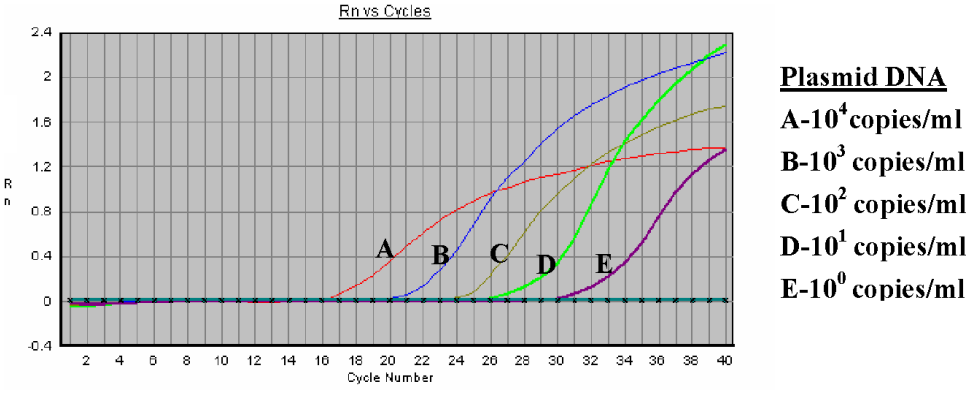


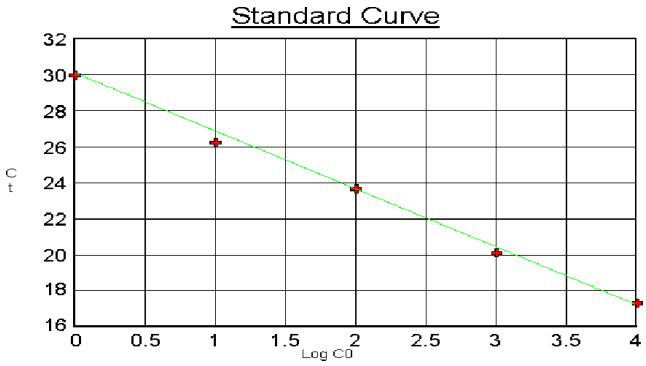
Figure 2. Plasma corticosterone in the control, chronic unpredictable stress (CUS), and Sini Tang groups following 21 days of CUS were shown. Plasma corticosterone level was higher in CUS group versus the control group ($p < 0.01$). Treatment with Sini Tang (2.5 and 10 g/kg) significantly countered CUS induced elevation of plasma corticosterone in a dose-dependent manner ($p < 0.05$ and $p < 0.01$, respectively). $^{+}$ and * indicates statistical significance in comparison to the control and CUS groups respectively and denotes $^{+},{}^{*}p<0.05$; $^{++},{}^{**}p<0.01$.

The present CUS procedure has been employed in several laboratories to investigate specific aspects of mood disorders, including both behavioral and physiological changes (Grippo *et al*., 2003; Solberg *et al*., 1999). In these experimental conditions, there has been a significant reduction of sucrose preference in CUS group compared with the control, which was

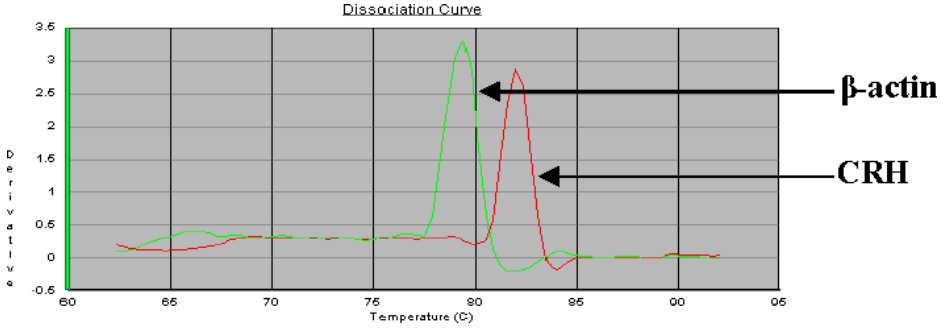


(A)

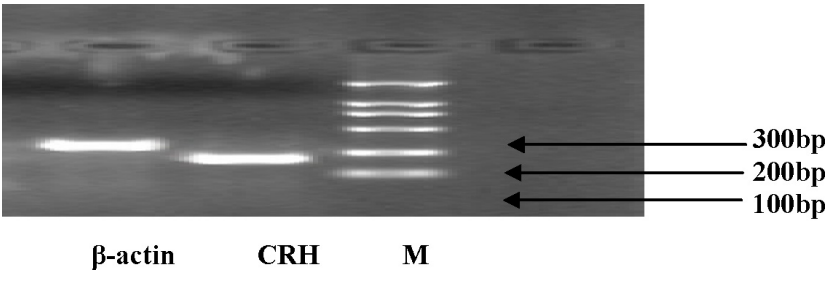
Figure 3. Real-time quantiative PCR analysis. A and B. Linear stand curve from plasmid DNA concentrations of CRH; C. Melting curves of the amplification products from CRH and β-actin; D. Gel electrophoretic analysis. Cytokines mRNA-specific DNA bands were identified by analyzing the real-time PCR products on 1.5% agarose gel. M, Molecular marker; predicted lengths of the PCR products were 239bp (β-actin), 184bp (CRH).



(B)



(C)



(D)

Figure 3. (*Continued*)

reduced to approximately 30% 21 days after the beginning of stress exposure; the reduction was comparable in all groups of stressed animals. These results demonstrate an operational change in reward sensitivity associated with CUS. CUS rats also exhibited anxious behavior as evidenced by decreased ambulation, rearing, increased grooming and immobility period in comparison to control rats. Thus, this animal model of depression seems indeed to meet the validity criteria necessary to validate an animal model of depression (Willner and Mitchell, 2002).

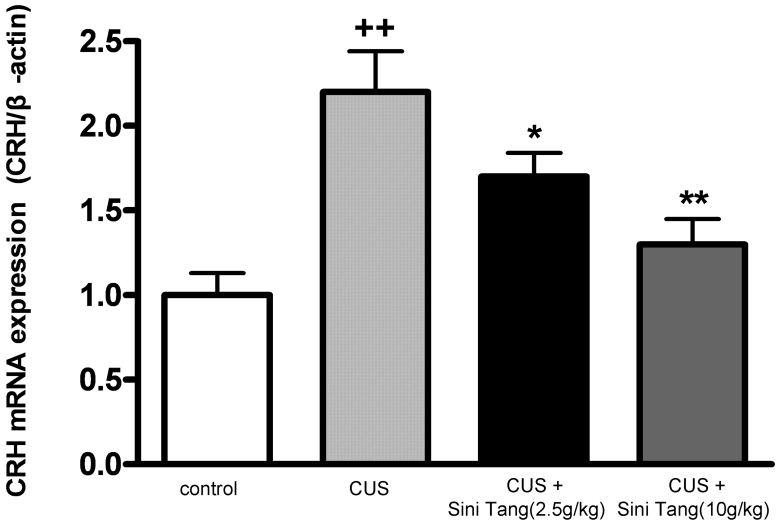


Figure 4. CRH mRNA expression in the control, chronic unpredictable stress (CUS), and Sini Tang groups following 21 days of CUS were shown. CRH mRNA expression was higher in the CUS group versus the control group ($p < 0.01$). Treatment with Sini Tang (2.5 and 10 g/kg) significantly suppressed CUS induced elevation of CRH mRNA expression in a dose-dependent manner ($p < 0.05$ and $p < 0.01$, respectively). $^{+}$ and * indicates statistical significance in comparison to the control and CUS groups respectively and denotes $^{+,*}p < 0.05$; $^{++,**}p < 0.01$.

During chronic stress, prolonged or repeated activation of the HPA axis can lead to long-term changes in HPA tone and responsiveness. In particular, chronic stress can lead to potentiated basal ACTH and/or corticosterone secretion, adrenal hypertrophy, and elevated CRH mRNA and protein expression (Dayas *et al*., 2000; Bauer *et al*., 2001; Gaab *et al.,* 2003). Consistent with previous work, this study indicated that chronic stress elevated plasma corticosterone and CRH mRNA level in the hypothalamus, which shows that the HPA axis is disorganization after exposure to CUS. A loss of HPA axis inhibition would generate greater HPA activity following each stress exposure, thereby resulting in exacerbated pathologies of chronic stress (Suemaru *et al*., 1995; Gold and Chrousos, 2002).

In this study, Sini Tang could augment the stress induced decrease in sucrose preference, and reverse the stress induced behavioral alteration in a dose-dependent manner. It could also suppress the CUS induced elevation in corticosterone and CRH mRNA level in a dose-dependent manner. These data provide direct evidence for Sini Tang treating depression.

Sini Tang has been widely used to treat *Yang*-deficiency, such as cold-bloodedness and cold-fear. *Yin-yang* theory is an essential part of TCM in diagnosis and treatment based upon overall analysis of symptoms and phenotypes of the body. *Yin-yang* balance is the optimal state of life owing to the equilibrium of *yin-yang* counteraction within a narrow range of variation, caused by the interplay, interdependence, transformation and suppression of each other between the counterparts of *yin-yang*. *Yang*-deficiency involved in the *yin-yang* imbalance points at the poor functional condition showing various symptoms such as lassitude and impotence, cold-bloodedness and cold-fear, lumbago and fatigue (Mahdihassan, 1989).

In fact, accumulated clinical observations demonstrated that*yang*-deficient patients diagnosed based on their symptoms and complaints showed HPA axis disorganization. Exposure to CUS induces the HPA axis disorganization results in suffering from the typical *yang*-deficient symptoms with reduced activity. Therefore, CUS may disturb the rhythm of HPA axis action and consequently result in disorders of the endocrine system, which shows the *Yang*-deficient symptoms. Sini Tang could treat *Yang*-deficiency and can help restore the *Yin-yang* balance.

In summary, this study is the first to describe the mechanisms of Sini Tang in treating CUS induced depression-like symptoms. However, Sini Tang has been widely used to treat *Yang*-deficiency, as the impaired T-cell cytokine production is involved in the *yang*-deficient process (Yao *et al*., 2007). Therefore, the effects of Sini Tang on T-cell cytokine expressions following CUS need to be investigated in further studies.

Acknowledgments

This study was supported by research grants from the National Natural Science Foundation of China (No. 30800301) and the Young Scientist Project from Institute of Psychology, Chinese Academy of Sciences (08CX043004).

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