Screening of the antidepressant-like effect of the traditional Chinese medicinal formula Si-Ni-San and their possible mechanism of action in mice

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ABSTRACT

Background: The traditional Chinese medicine formula Si-Ni-San has well therapeutic applications in improvement of mental diseases including depression. However, the neuropharmacological and neuroendocrine mechanisms of the formula on antidepressant-like action have not been reported. **Objective:** Herein, we explored the antidepressant-like effect and its mechanism of Si-Ni-San. **Materials and Methods:** Acute effect of Si-Ni-San on the immobility time was assessed in the mouse forced swim test (FST) and tail suspension test (TST). Moreover, we investigated the neurochemical, neuroendocrine, and neurotrophin systems involved in the antidepressant-like effect of this formula. **Results:** Si-Ni-San significantly decreased the immobility time after acute treatment in the mouse TST (1300 mg/kg) but not in the FST compared with the control group. In addition, pretreatment of mice with PCPA or AMPT prevented the anti-immobility effect of Si-Ni-San (1300 mg/kg) in the TST. Moreover, acute Si-Ni-San (1300 mg/kg) decreased serum corticosterone levels, elevated serotonin (5-HT), norepinephrine (NE), and dopamine (DA) levels without affecting brain-derived neurotrophic factor (BDNF) levels in the whole brain exposed to TST. **Conclusion:** The acute antidepressant-like action of Si-Ni-San is mediated by the monoaminergic and neuroendocrine systems although underlying mechanism still remains to be further elucidated, and this formula should be further investigated as an alternative therapeutic approach for the treatment of depression.

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INTRODUCTION

Nowadays, depression with growing danger has become a big health problem all around the world. Recent studies indicated that about 30% of depressive patients failed to respond satisfactorily to commercially available antidepressants in mono-therapy.[1] With the requirement of more therapeutic efficacy and less adverse effect, a renewed interest has been generated in traditional Chinese medicine (TCM), traditional Ayurvedic medicine, and other folk medicines.[2-4] Among these methods, TCM is one of the most ancient traditions with sound rationale and a wide range of applications. TCM formulas are commonly used by folk doctors to assist Chinese people in dealing with various diseases. Si-Ni-San, a famous TCM formula, comprising Radix Bupleurichinensis (Chaihu), Radix Paeoniae Alba (Shaoyao), FructusCitriAurantii (Zhishi), and Radix GlycyrrhizaeUralensi (Gancao), in a ratio of 1:1:1:1, was recorded in the ancient manuscript of "Shang-Han Lun," written one thousand years ago by the famous Chinese folk doctor Zhong-Jing Zhang. Recently, Si-Ni-San has been shown to alleviate irritable bowel syndrome of the constipation predominant type, decrease palmoplantar perspiration in palmoplantarhidrosis, reduce blood glucose, and improve insulin resistance.[5-7] In addition, it is mainly empirically advocated for improvement of mental diseases including depression and other conditions.[8,9] In animal experiment, Si-Ni-San treatment has an antidepressant-like effect in antagonizing chronic restraint stress-induced stress-related disorders.[10] However, the detailed mechanisms of the formula on antidepressant-like action have not been reported.

It is well-known that monoamine depletion in synaptic

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clefts in the central nervous system plays a key role in the pathophysiology of depression. As a result, the mechanism of antidepressant drugs has been investigated on the basis of monoamine systems.[11] Evidence suggests that increasing brain monoamine neurotransmitters is an effective way to treat depression.[12] In addition, numerous studies found that corticosteronehypersecretion and brain-derived neurotrophic factor (BDNF) down-expression was associated with depression, and in fact was served as a depression marker and a target for antidepressants.[13,14]

Considering the therapeutic application of Si-Ni-San in the improvement of mood disorders in clinic, the present work sought to investigate the effect of Si-Ni-San in forced swimming test (FST), tail suspension test (TST), and open-field test (OFT). Additionally, the involvement of the monoaminergic, neuroendocrine, and neurotrophin systems in its antidepressant-like action through the use of pharmacological procedures was also investigated.

MATERIALS AND METHODS

Preparation of extracts of Si-Ni-San and other drugs

Traditional Chinese medicines Radix BupleuriChinensis (Chaihu), Radix Paeoniae Alba (Shaoyao), Fructus Citri Aurantii (Zhishi), and Radix Glycyrrhizae Uralensi (Gancao) were purchased from Xiamen Medicine Station and botanically authenticated by Cheng-Fu Li (Xiamen Hospital of Traditional Chinese Medicine) as *Bupleurumchinense* DC. (Voucher specimen number HU/CE-12291), *Paeoniaalbiflora* Pall. (HU/CE-12292), *Citrus aurantium* L. (HU/CE-12293), and *Glycyrrhizauralensis* Fisch. (HU/CE-12294), respectively. Si-Ni-San extracts were extracted as described by Chen *et al.*[15] Briefly, all the herbs were chopped intosmall pieces and ground. The powdered mixture Si-Ni-San (400 g) [Radix Bupleuri Chinensis (100 g), Radix Paeoniae Alba (100 g), Fructus Citri Aurantii (100 g), and Radix Glycyrrhizae Uralensi (100 g)] was immersed in 2400 mL distilled water for 30 min, then extracted for 30 min (100°C). The residues were extracted with 2000 mL distilled water for 30 min (100°C) again. Finally, the two supernatants were combined and filtered through atwo-layer mesh. The water extract was concentrated in vacuo and lyophilized into powders. The yield was 83.4 g [yield 20.85% (w/w)] for the water extracts of Si-Ni-San.

p-chlorophenylalanine methyl ester (PCPA), α-methyl-*p*-tyrosine (AMPT), serotonin (5-HT), and dopamine (DA) standards were purchased from Sigma-Aldrich Co. Norepinephrine (NE) standard was obtained from the National Institute for the Control of Pharmaceutical and Biological Products. Fluoxetine hydrochloride was purchased from Changzhou Siyao Pharmaceuticals Co., Ltd. All other chemicals were of high-purity analytical grade obtained from Shanghai Chemical Reagent Co., Ltd.

Animals

Male Kunming mice (20-24 g) were purchased from Laboratory Animal Centre, Fujian Medical University, Fujian Province, P. R. China. Animals were housed 5 per cage (320×180×160 cm) under a normal 12-h/12-h light/ dark schedule with the lights on at 07:00 a.m. and had free access to tap water and food pellets. Ambient temperature and relative humidity were maintained at 22±2°C and at 55±5% and were given a standard chow and water *ad libitum* for the duration of the study. The animals were allowed 1 week to acclimatize themselves to the housing conditions before the beginning of the experiments. The experiments were performed with 10 mice per treatment group according to a randomized schedule. All procedures were performed in accordance with the guidelines of the China Council on Animal Care (Regulations for the Administration of Affairs Concerning Experimental Animals, approved by the State Council on October 31, 1988 and promulgated by Decree No. 2 of the State Science and Technology Commission on November 14, 1988).

Drug treatments

The dosage of Si-Ni-San for human adults is 24 g (the total raw materials), equivalently for mice, this dosage is 3120 mg/kg calculated by the formula that converts dosage of human into that of mouse according to the respective body surface areas. In the present study, the yield of Si-Ni-San water extracts was 20.85%, making the dosage of Si-Ni-San extract 650 mg/kg. Si-Ni-San, fluoxetine, and PCPA were dissolved in 0.9% physiological saline, and AMPT was suspended in saline with 10% (v/v) Tween-80 (polyoxyethylenesorbitanmonooleate). The tests were performed between 11:00 a.m.-13:00 p.m.

Experiment 1

Animals were divided into five experimental groups: one 0.9% saline control group, one fluoxetine group (20 mg/kg, p.o.),[16] and three Si-Ni-San treatment groups (325, 650, and 1300 mg/kg, p.o.). The administration volume was 10 mL/kg-body weight. To investigate the antidepressant-like effect of Si-Ni-San, Si-Ni-San or vehicle was administrated by oral route 60 min before FST, TST, or OFT. Independent groups of animals were tested in each behavioral test.

Experiment 2

To assess the possible involvement of the serotonergic and noradrenergic system in the antidepressant effect of Si-Ni-San in the TST, mice were pretreated with PCPA (100 mg/kg, i.p., an inhibitor of serotonin synthesis) or vehicle, once a day for 4 consecutive days.[17] Then, 24 hrs after the last PCPA or saline injection, animals were treated with Si-Ni-San (1300 mg/kg, p.o.) or vehicle and were tested in the TST 60 min later.

Experiment 3

To investigate the possible involvement of the noradrenergic system in the antidepressant-like effect of Si-Ni-San in the TST, animals were pretreated with AMPT (100 mg, i.p., an inhibitor of the enzyme tyrosine hydroxylase) once.[17] After 4 hrs, they received Si-Ni-San (1300 mg/kg, p.o.) or vehicle and were tested in the TST 60 min later.

Forced swim test

The Forced swim test (FST) used was the same as described in detail elsewhere,[18] with some modification. Briefly, mice were individually placed in a glass cylinder (20 cm in height, 14 cm in diameter) filled with 10-cm high water (25±2°C). All animals were forced to swim for 6 min, and the duration of immobility was observed and measured during the final 4 min interval of the test. The immobility period was regarded as the time spent by the mouse floating in the water without struggling and making only those movements necessary to keep its head above the water. A competent observer blind to treatment scored the videotapes.

Tail suspension test

The Tail suspension test (TST) was conducted as previously described.[19] Briefly, mice were individually suspended by tail with a clamp (1 cm from the tip of the end) in a box (25 × 25 × 30 cm) with the head 5 cm from the bottom. Testing was carried out in a darkened room with minimal background noise. Mouse was suspended for a total of 6 min, and the duration of immobility was observed and measured during the final 4 min interval of the test. Mice were considered immobile only when they hung passively and completely motionless. A competent observer blind to treatment scored the videotapes.

Open-field test

The locomotor activity was assessed in an Open-field test (OFT) according to the method ofMao *et al.*[20] The apparatus consisted of a wooden box measuring 40×40×30 cm, with the floor divided into 25 equal squares (8 × 8 cm). The number of squares crossed with all paws (crossing) was counted in a 6-min session.

Determination of monoamine neurotransmitter levels

Animals were decapitated after the TST in experiment 1. Mouse brain monoamine neurotransmitters 5-HT, NE, and DA levels were measured by HPLC-ECD.[21,22] The whole brains were quickly removed and stored at -80°C until assayed. The brain tissues were homogenized in an ice-cold solution of 0.4 M perchloric acid (6.6 uL/mg) containing 5 mM sodium bisulfite and 0.04 mM EDTA for avoiding oxidation, and then centrifuged at 15 000×*g* for 15 min at 4°C. Standard solution or sample was injected into the DIKMA C-18 column (5 μm, 150 ×4.6 mm). The separation was done in an isocratic elution mode at column temperature 20°C, using a mobile phase consisting of 17.6% methanol (v/v) and 82.4% distilled water containing EDTA (0.0876 mM), triethylamine (1.512 mM), (1S)-(+)-10-camphorsulfonic acid (9 mM), $Na_2HPO_4 \cdot 12H_2O$(20 mM), and citrate (15 mM), at a flow rate of 0.7 mL/min.The measurements were done at electrode potentials of a glassy carbon working electrode at +650mV versus Ag/ AgCl electrode. 5-HT, NE, and DA were identified and quantified by comparing their retention times and peak areas to those of standard solutions. The contents of 5-HT, NE, and DA were expressed in ng/g wet weight tissue.

Serum corticosterone assay

Blood was collected on ice and separated in a refrigerated centrifuge at 4°C. Serum was stored at-20°C until assays were performed. Serum corticosterone levels were measured using anenzyme immunoassay kit (Enzo Life Sciences).

Brain BDNF assay

Brain regions of frontal cortex and hippocampus samples were homogenized in lysis buffer containing 137mMNaCl, 20mMTris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1μg/ml leupeptin, 0.5 mM sodium vanadate. The homogenate was centrifugated at 16000 × g for 30 min at 4°C, and the supernatant was collected and stored at -80°C until assay. BDNF protein was measured using BDNF E_{max}ImmunoAssay System (Promega) according to the protocol of the manufacturer.

Statistical analyzes

All data were expressed as mean±S.E.M. To compare experimental and control groups, we used one or two-way analysis of variance (ANOVA), followed by *post-hoc* Dunnett's test. A value of $P<0.05$ was considered statistically significant for analysis.

RESULTS

Effect of Si-Ni-San on the immobility time in the FST and TST

A one-way ANOVA revealed a significant effect of the treatment only in the TST [$P<0.05$] but not in FST [Figure 1]. Post hoc analysis indicated a significant decrease in the immobility time elicited by the administration of Si-Ni-San at the doses of 1300 mg/kg [$P<0.05$] in the TST compared with the control group. Fluoxetine (20 mg/kg), the positive control, also reduced immobility time in the TST in mice [$P<0.05$, $P<0.05$, respectively].

In order to detect any association of immobility in the TST with changes in locomotor activity, mice treated with Si-Ni-San were tested in the OFT. Treatments with Si-Ni-San at 650 and 1300 mg/kg produced no significant difference in crossing number compared with the control group (data not shown).

Involvement of the monoaminergic system

The results depicted in Figure 2a showed that the pretreatment of animals with PCPA (100 mg/kg.) prevented the anti-immobility effect of the Si-Ni-San (1300 mg/kg) in the TST [$P<0.01$]. A two-way ANOVA showed significant differences for Si-Ni-San treatment [$P<0.01$], PCPA pretreatment [$P<0.05$], and Si-Ni-San × PCPA interaction [$P<0.05$].

The results depicted in Figure 2b showed that pretreatment with AMPT (100 mg/kg) prevented the anti-immobility effect of the Si-Ni-San (1300 mg/kg) in the mouse TST [$P<0.05$]. A two-way ANOVA indicated significant effect of Si-Ni-San treatment [$P<0.01$] and Si-Ni-San × AMPT interaction [$P<0.05$] without effect of AMPT pretreatment on the immobility time [$P> 0.05$].

Effect of acute Si-Ni-San treatment on brain monoamine neurotransmitter levels

Si-Ni-San at 1300 mg/kg resulted in significant increases in 5-HT, NE, and DA levels [$P<0.05$, $P<0.05$, $P<0.05$, respectively] in the whole mouse brain [Figure 3]. Fluoxetine (20 mg/kg), the selective serotonin reuptake inhibitor used as positive control in our study, only induced increase in 5-HT levels compared with the control group [$P<0.01$].

Effect of acute Si-Ni-San treatment on serum corticosterone levels

A one-way ANOVA revealed a slight but not significant effect [$P=0.065$] of the treatment in serum corticosterone levels exposed to TST. Post hoc test showed that Si-Ni- San at 1300 mg/kg [$P<0.05$] significantly reduced the serum corticosterone levels in mice compared with the control group. Treatment of mice with fluoxetine at 20 mg/kg did not affect the serum corticosterone [Figure 4].

Effect of acute Si-Ni-San treatment on brain BDNF protein levels

In analysis of BDNF levels in the brain of TST-induced mice, as compared to levels in vehicle mice, neither Si-Ni-San nor fluoxetine showed significant differences in the amount of BDNF levels [Figure 5].

DISCUSSION

The mouse FST and TST are the widely used behavioral models, which have a strong predictive validity and are used to screen and evaluate the efficacy of antidepressant drugs. In these models, the main indication of the antidepressant-like action of any given compound is the marked reduction in immobility time.[17,23] In the present

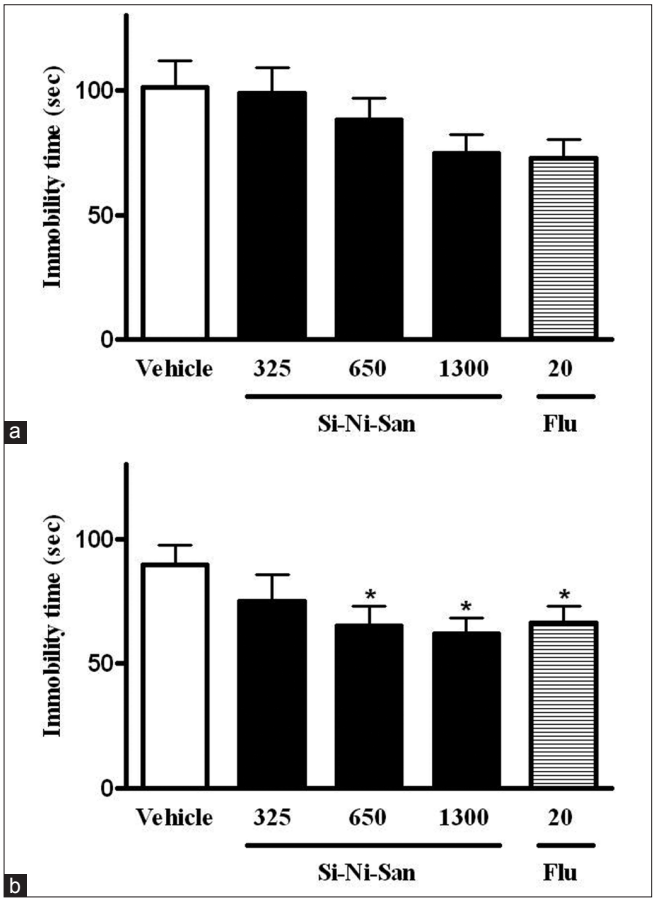

Figure 1: Effect of Si-Ni-Sanon the immobility time in the mouse FST (a) and TST (b). *$P<0.05$ vs. TST with vehicle group (Control)

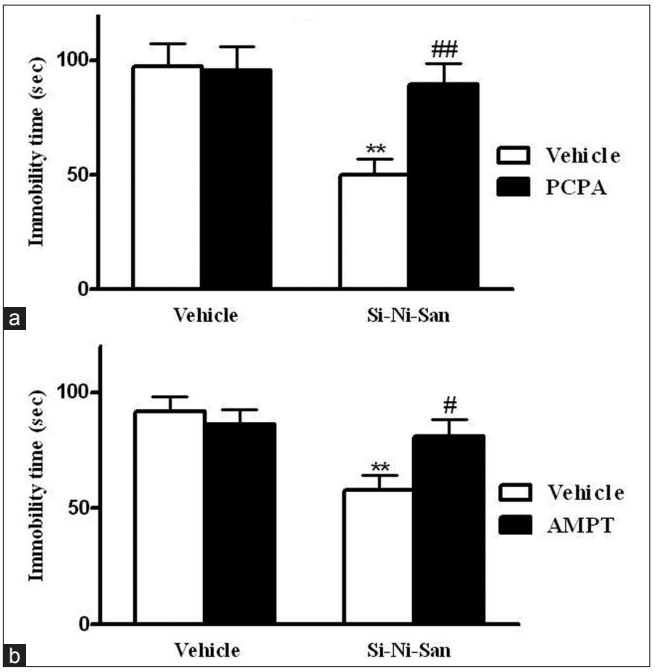

Figure 2 (a-b): Effect of pretreatment with PCPA (100 mg/kg, i.p. once a day for 4 consecutive days) and AMPT (100 mg/kg, i.p.) on the Si-Ni-San (1300 mg/kg, p.o.)-induced reduction in immobility time in the mouse TST. $P<0.01$ vs. TST with vehicle group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Si-Ni-San group pretreated with vehicle

study, our results demonstrated that acute administrations of Si-Ni-San produced a significant reduction in immobility time in the mouse TST (650, 1300 mg/kg, p.o.) and FST (1300 mg/kg, p.o.).

To avoid false positive effect in the FST and TST, we used the OFT to exclude the anti-immobility effect from psycho-stimulant effect. At the doses those significantly improved antidepressant performance, Si-Ni-San were unable to affect crossing number in the mouse OFT. Thus, antidepressant-like action of Si-Ni-San seemed unlikely to be due to an increase in locomotor activity.

In addition, it was noteworthy that antidepressant-like effect of Si-Ni-San was observed more in the TST than in the FST in mice. The antidepressant-like effect of the acute Si-Ni-San treatment was observed at a lower dose in the TST (650 mg/kg, p.o.) than in the FST (1300 mg/kg, p.o.). The underlying principle measuring the lack of active coping behavior is identical in the two behavioral models in mice, but their variability in response to certain antidepressants indicates potentially different substrates and neurochemical pathways mediating performance in these tests.[24,25] These issues may underlie the observed behavioral differences in the present study.

Depression is a serious emotional disorder with high morbidity and mortality,[26] which is the result from a complex interaction among behavioral, neurobiological, and other physiological factors.Especially, depression has been associated with disturbances of brain monoamine neurotransmitters 5-HT, NE, and DA activity, and data concerning the neurotransmitters variations in depression have probably been the most widely studied. Commercially available antidepressants that increase brain 5-HT, NE, and DA levels have been shown to alleviate effectively the symptoms of depression.[27,28] Thus, serotonergic, noradrenergic, and dopaminergic systems play the major roles in the actions of antidepressant drugs.[28,29]

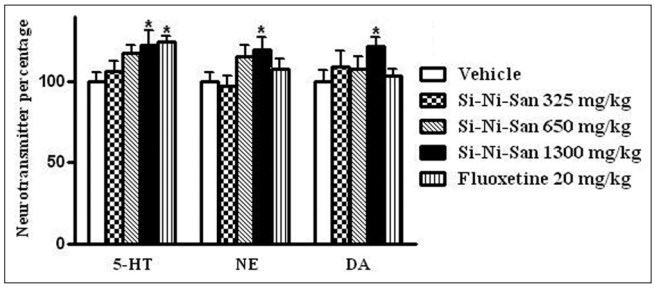

Figure 3: Effect of Si-Ni-San on the monoamine neurotransmitter levels in the whole brain in the mice exposed to the TST. Data were expressed as percentages with respect to vehicle control group. The basal values of these neurotransmitters are 5-HT (710.0±40.2 ng/g), NE (403.0±22.2 ng/g), and DA (918.6±63.8 ng/g). **P*<0.05 vs. TST with vehicle group (Control)

The identification of specific monoamine systems in the brain that become functionally active in response to antidepressant drugs has led to a clearer understanding of how the drugs improve the central system. Thus, in the present study, we investigated the influence of the pretreatment with the inhibitor of the enzyme tryptophan hydroxylase, PCPA, in the antidepressant-like effect of Si-Ni-San in the TST. It was confirmed that treatment with PCPA caused a rapid relapse in depressed patients who had responded to the antidepressant medication.[30] PCPA treatment only significantly decreased 5-HT levels in the brain while it did not affect NE and DA levels.[31] Depletion of 5-HT levels by PCPA completely blocked reductions of immobility by the selective Serotonin Reuptake Inhibitors (SSRIs) such as fluoxetine and did not alter the behavioral effect of the norepinephrine reuptake inhibitors (NRIs) such as desipramine.[32] Our results showed that pretreatment with PCPA significantly inhibited the anti-immobility effect of Si-Ni-San in the mouse TST. Thus, it suggests that the antidepressant-like effect of Si-Ni-San might be mediated by the serotonergic system.

Although 5-HT is definitely implicated in depression, it is necessary to investigate the role of NE and DA in depression.[30] We also evaluated the influence of the pretreatment with AMPT in the antidepressant-like effect of Si-Ni-San in the TST. AMPT is an inhibitor of the rate-limiting enzyme of catecholamine synthesis and tyrosine hydroxylase and disturbs both NE and DA synthesis.[33] The production of NE is dependent on a conversion of tyrosine to L-dopa by the enzyme tyrosine hydroxylase. This process can be inhibited by AMPT treatment. As a result, AMPT treatment significantly decreased brain NE and DA levels, whereas it did not affect 5-HT levels.[34] Depressed patients with NRIs-treated depression have a transient return of depressive symptoms following AMPT administration.[35] AMPT-

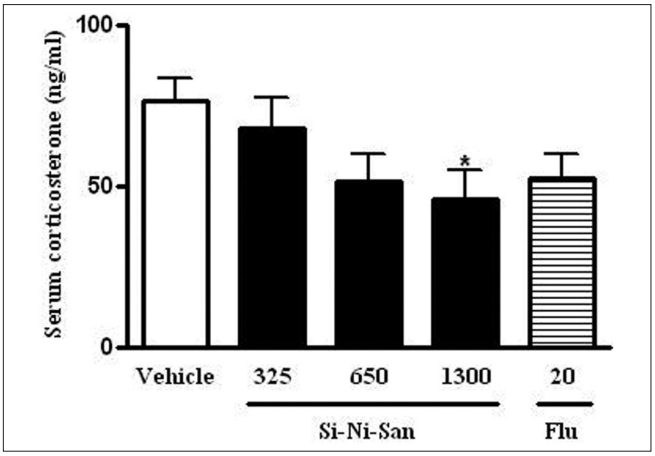

Figure 4: Effect of Si-Ni-San on the serum corticosterone levels in the mice exposed to the TST. **P*<0.05 vs. TST with vehicle group (Control)

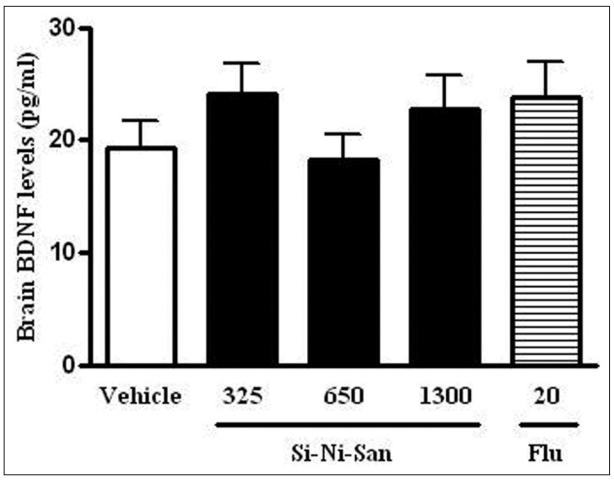

Figure 5: Effect of Si-Ni-San on the brain BDNF levels in the mice exposed to the TST

induced return of depressive symptoms was experienced by 11 of the 18 patients and led to decreased brain metabolism in a number of cortical areas, with the greatest magnitude of effect in prefrontal cortex and thalamus.[36] In the present study, the pretreatment of mice with AMPT (100 mg/kg, i.p.) was able to prevent the anti-immobility effect of Si-Ni-San in TST. Thus, it suggests that the noradrenergic and/or dopaminergic system might be implicated in the antidepressant-like effect of Si-Ni-San.

To support the hypothesis that the antidepressant-like effect of Si-Ni-San is mediated by the increase of monoamine neurotransmitters, the effect of Si-Ni-San on 5-HT, NE, and DA levels in mouse brain was studied. Our results showed Si-Ni-San administration produced a marked increase of 5-HT, NE, and DA contents in the whole brain, which is consistent with the behavioral changes exhibited in the pharmacological interaction model (pretreatment with PCPA and AMPT in the TST).

Besides monoamine deficiency, hypersecretion of glucocorticoids is also involved in the pathogenesis of depression.[37] Glucocorticoids (principally cortisol in humans and corticosterone in rodents), a vital steroid hormone released in response to stress, are markedly higher than those of controls in depressed patients and stress-induced depression-like rodents.[13,38] Our results showed that Si-Ni-San alleviated the corticosterone elevation by TST, in a way similar to previous literature obtained using fluoxetine, amitriptyline, and mitragynineinmice exposed to TST.[39]

Recent study indicates that BDNF regulation is involved in the mechanisms of action of antidepressant drugs.[40] However, our study failed to find any significant difference in BDNF levels in brain following Si-Ni-San treatment. As previous study reported that one explanation for the delayed response (take weeks to months to achieve remission) by clinical antidepressant may be a need for physical growth and reorganization in the brain responses, which were mediated by BDNF signaling.[41] Therefore, we speculated that chronic treatment was required to induce an increase in BDNF levels by Si-Ni-San since that the time of drug treatment and animal sacrifice has been shown to play an important role in the regulation of BDNF expression.[42,43]

An acute administration to evaluate the antidepressant-like effect of Si-Ni-San in the two experimental models has been performed in this study, but the clinical effect of antidepressants appears after several weeks post-treatment. Therefore, further studies with chronic treatment will be taken to reveal the long-term antidepressant-like effect of Si-Ni-San.

CONCLUSION

Taken together, our study confirmed that the famous TCM formula Si-Ni-San possessed the antidepressant-like effect both in the mouse TST but not FST, without differences of locomotor activities in the OFT. In addition, pretreatment with PCPA or AMPT significantly inhibited anti-immobility effect of Si-Ni-San in the TST. Moreover, acute Si-Ni-San treatment produced a marked increase of brain 5-HT, NE, and DA levels as well as a decrease of serum corticosterone levels but did not affect brain BDNF levels in mice exposed to TST. These results suggested that Si-Ni-San possessed antidepressant-like property that was mediated via the monoaminergic and neuroendocrine systems. Thus, the present study provided a basis for clinical application of the ancient TCM formula Si-Ni-San.

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