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ORIGINAL RESEARCH

The depressive-like behaviors of chronic unpredictable mild stress-treated mice, ameliorated by Tibetan medicine Zuotai: involvement in the hypothalamic–pituitary–adrenal (HPA) axis pathway

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Background: Zuotai, a famous Tibetan medicinal mixture containing metacinnabar, is traditionally used for the purpose of tranquilizing minds and soothing nerves. However, it still lacks substantial experimental data for it to be approved for use.

Aim: This study was designed to assess the effects of Zuotai on depressive-like symptoms in a chronic unpredictable mild stress (CUMS) mouse model, and to explore its potential mechanism, particularly the hypothalamic–pituitary–adrenal (HPA) axis pathway.

Materials and methods: First, Kunming mice were exposed to the CUMS procedure and simultaneously administered Zuotai or imipramine (positive control) by gavage continuously for 6 weeks. Then, depressive-like behaviors of mice in each group were tested with the sucrose preference test, forced swimming test, tail suspension test, and open field test. Meanwhile, the three key neuroendocrine hormones (corticotropin releasing hormone, adrenocorticotropic hormone and corticosterone) in HPA axis pathway, and the level of the emotion-related monoamine neurotransmitters (5-hydroxytryptamine and norepinephrine) were measured using enzyme-linked immunosorbent assay. Furthermore, total mercury in the hypothalamus and hippocampus were determined using an automatic, direct mercury analyzer.

Results: Zuotai or imipramine significantly increased the body weight and the sucrose preference ratio in sucrose preference test, and dramatically improved motor activity in forced swimming test, tail suspension test, and open field test in CUMS mice. Zuotai or imipramine remarkably decreased levels of corticotropin-releasing hormone, adrenocorticotropic hormone, and corticosterone in the HPA axis, and increased levels of 5-hydroxytryptamine and norepinephrine in the serum in CUMS mice. However, a small amount of mercury was deposited in the hypothalamus and hippocampus in Zuotai-treated mice, which may pose a potential risk to the central nervous system.

Conclusion: Zuotai has a strong ability to ameliorate depressive-like behaviors in CUMS-treated mice through inhibition of the HPA axis and upregulation of monoamine neurotransmitters. These findings provide new insight into the pharmacological effect of Zuotai on depression.

Keywords: Tibetan medicine, Zuotai, HgS, depression, chronic unpredictable mild stress, CUMS, HPA axis

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Introduction

Depression is a global mental disorder that has high incidence, high recurrence, and high self-mutilation and suicide rates.[1,2] Clinically, depression is mainly characterized

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by persistent depressed mood, loss of interest and enjoyment, anxiety, a significant reduction in volitional activity, cognitive impairment, mental retardation, and other symptoms.[3–5] According to the World Health Organization,[6] an estimated 350 million people of all ages currently suffer from depression around the world, and more than 800,000 people commit suicide each year. Moreover, depression is expected to be one of the top two leading causes of disability-adjusted life years in 2020. Currently, the effective remission rate of antidepressants ranges from 60% to 80%, and the rate of favorable prognosis is approximately 30%.[7–9] Therefore, there is an urgent need to develop new treatment strategies that can significantly improve outcomes. Traditional or natural medicine is one of the most important sources for new antidepressants.[10]

In many traditional medicine systems, mercuric sulfide (HgS) has been used to treat various diseases across thousands of years.[11] Zuotai, a famous Tibetan medicinal mixture containing 54.5% metacinnabar (β-HgS), has been used for tranquilizing the mind, soothing the nerves, invigorating the spleen, strengthening the body, and reducing the toxicity while increasing the efficacy of other drugs.[12–15] In the clinic, Zuotai is usually utilized as a core ingredient in many classic compound preparations such as Qishiwei Zhenzhu Wan, Renqing Changjue, Renqing Mangjue, and so on.[16,17] Indeed, previous studies have provided a number of evidences about Zuotai to support its traditional medicinal use.[18–22] Some studies have preliminarily confirmed the tranquilizing and sedative functions of Zuotai, which could shorten sleep induction time and increase sleep time in response to pentobarbital or chloral hydrate, reduce the convulsion rate due to strychnine, and prolong convulsion due to strychnine and nikethamide in mice.[19,21] However, the scientific evidence on this is still scarce.

There is a subset of clinical depression that appears to be related to hypothalamic–pituitary–adrenal (HPA) axis dysfunction.[23–25] It has been reported that chronic adverse stress could trigger oversecretion of three core factors (corticotropin-releasing hormone [CRH] by hypothalamus, adrenocorticotropic hormone [ACTH] by pituitary and glucocorticoid [GC] by adrenal cortex) in the HPA-axis pathway, resulting in hyperactivity of HPA-axis function, thereby establishing a vicious cycle;[26,27] furthermore, the hyperactivity of the HPA axis could reduce the synthesis of central monoamine neurotransmitters such as 5-hydroxytryptamine (5-HT) and norepinephrine (NE), as well as damage hippocampal neurons, ultimately inducing depression.[26,28] Meanwhile, effective antidepressive treatment could restore HPA-axis dysfunction to its normal state.[29]

The chronic unpredictable mild stress (CUMS) model is a classic, established model for researching depressive disorders and antidepressants.[30] The chronic mild stress paradigm resembles the etiology of some human depression disorders.[30,31] Moreover, this model can recapitulate some major symptoms of human depression.[30,32] Therefore, in the present study, the CUMS model of Kunming albino mice was used to evaluate antidepressive effects of Zuotai by conducting the sucrose preference test (SPT), forced swimming test (FST), tail suspension test (TST), and open field test (OFT). Neuroendocrine factors in the HPA axis and monoamine neurotransmitters in serum were detected to reveal the potential antidepressant mechanism of Zuotai. Total mercury in the hypothalamus and hippocampus was measured to show the potential risk of heavy metal accumulation in the brain.

Materials and methods

Ethics approval

The Animal Experimentation Committee of Northwest Institute of Plateau Biology, Chinese Academy of Sciences approved the protocol for all animal experiments conducted in this study. Procedures involving mice and their care were conducted in conformity with international guidelines, the European Community guidelines (EEC Directive of 1986; 86/609/EEC), and the US guidelines (NIH Publication #85-23, revised in 1985).

Preparation of Zuotai

Zuotai (No 20110705) was prepared by the Company of Tibetan Medicine in the Tibetan Autonomous Region using the following ingredients: 5 kg metallic mercury, 5 kg sulfur, 0.333 kg Nengchi Eight Metal ash (gold, silver, bronze, copper, brass, iron, lead, and tin, all in equal weight proportions), 0.333 kg of Nengchi Eight Minerals ash (gold ore, silver ore, magnet ore, tufa, pyritum, orpiment, realgar, and red mica, all in equal weight proportions), and other auxiliary materials, according to a China patent(88107006.8).[12] The producing mixture was designated as ZT20110705 and deposited at the Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation.

Metallic mercury and sulfur were initially treated by special procedures (eg, washing, refining, detoxifying), then pooled together, and ground into a black powder. The Nengchi Eight Metals and the Nengchi Eight Minerals were also burnt into ashes through complex processes. Finally, the quicksilver powder, Nengchi Eight Metals ashes, and Nengchi Eight Minerals ashes were completely ground into a black fine powder and designated as Zuotai (No 88107006.8,

China Patent).[12] Synchrotron radiation X-ray absorption fine structure (SR-XAFS), X-ray diffraction (XRD), X-ray fluorescence spectroscopy (XRF), scanning electron microscopy (SEM), and atomic force microscopy (AFM) were employed to verify the quality of the Zuotai. Details of their physicochemical characteristics are published elsewhere.[17,33]

Reagents and instruments

Imipramine (purity: 98%; 3B Scientific Co., Libertyville, IL, USA) and the enzyme-linked immunosorbent assay (ELISA) kits for Cort and ACTH were purchased from Nanjing Jiancheng Bioengineering Institute (Ningjing, Jiangsu, People's Republic of China); ELISA kits of CRH, 5-HT, and NE were purchased from Cloud-Clone Corp. Sucrose (AR) was purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). NaCl (PT), $NaH_{2P}O_4$ (AR), KH_2PO_4 (AR), KCl (AR), absolute ethyl alcohol (AR), and starch (FG) were purchased from Tianjin Fuyu Fine Chemical Co., Ltd. (Tianjing, People's Republic of China).

The following instruments were used in the present study: Open field experiment system equipment (OFT-100, Chengdu Techman Software Co., Ltd., Sichuan, People's Republic of China), multi-wavelength microplate reader (Enspire2300, Perkin Elmer Co., Ltd., USA), HD digital video recorder (GZ-MG174AC, Sony Co., Japan), automatic direct mercury analyzer (DMA-80, Milestone Co., Ltd., Italy), UP water purification system (Milli-Q Reference, Millipore Co., Ltd., USA), ultra-low temperature freezer (REVCO Ult-2586-4-v, Thermo-Fisher Co., Ltd., USA), electronic balance (0.0001 g, ME204, Mettler Toledo Co., Ltd., Switzerland), pH meter (PB10, Sartorious Co., Ltd., Germany), high-speed freezing centrifuge (Sigma 3K-15, Sigma Company, Germany), constant temperature magnetic stirrer (85-2A, Jintan Scientific Analytical Instrument Co., Ltd., People's Republic of China), high-throughput tissue grinder (Scientz-48, NingBo Scientz Biotechnology Co., Ltd., People's Republic of China), micropipettor (Thermo Electron Co., Ltd., People's Republic of China), and manual counter (PC3860, Shenzhen Huibo Industry & Trade Co., Ltd., People's Republic of China).

In addition to this, according to previous reports, the suspended tail test system equipment[34] and the forced test system equipment[35] were created and used by our laboratory in this study with minor revision.

Experimental animals

Male, random-bred, Kunming albino mice (age 4–5 weeks; mean weight [mean ± SD] 22–25 g) were used in carrying out the studies. These mice (SCXK [Gan] 2015-0002) were purchased from the Gansu University of Traditional Chinese Medicine, Lanzhou, People's Republic of China.

Housing and husbandry

The Kunming mice were housed in the laboratory animal facility of Northwest Institute of Plateau Biology, Chinese Academy of Sciences, under specific pathogen-free conditions, and maintained with a 12-h light/dark cycle (lights on at 8:00 am) at 22°C–25°C. Mice growth-maintenance feed and sawdust pads were purchased from Beijing Ke'ao Xieli Feed Co., Ltd. (Beijing, People's Republic of China). The animals had free access to standard diet and water. The Animal Experimentation Committee of Northwest Institute of Plateau Biology, Chinese Academy of Sciences approved the protocol for all animal experiments conducted in this study. The procedures involving mice and their care were conducted in conformity with international guidelines, the European Community guidelines (EEC Directive of 1986; 86/609/EEC), and the US guidelines (NIH Publication #85-23, revised in 1985). All sections of this report comply with ARRIVE guidelines for reporting animal research.

Pharmacological treatments and administration procedures

CUMS procedure

The CUMS procedure was carried out according to the method described by Willner and Katz,[30,36] with minor modifications. Briefly, these stress methods include the following: 1) food deprivation for 24 h; 2) drinking water deprivation for 24 h with no drink bottle; 3) cage tilted at a 45° angle for 24 h; 4) placed together as a group for 2 h, then individually separated; 5) application of restraint stress for 2 h; 6) lighting at night for 12 h; 7) clamping of tail for 15 min; 8) forced swimming in cold water (4°C–8°C) for 5 min; 9) exposure to a foreign environment for 24 h; and 10) empty drink bottle. The above stress methods were randomly applied each day for 6 weeks consecutively; the same stress method was not continuously applied to ensure the mice would be unable to anticipate the next type of stress that would be applied.

Drug administration

Zuotai and a positive drug (imipramine, IMI) were suspended or dissolved in 2% starch water solution. Male mice were allowed to acclimate to their surroundings for 7 days prior to initiation of the experiments. Sixty mice were randomly allocated to one of the following six groups (10 mice for each group): control (2% starch solution), CUMS+Veh (CUMS +2% starch solution), CUMS+Zuotai I (CUMS +6.06972 mg·kg^{-1}

Zuotai suspended in 2% starch solution), CUMS+Zuotai II (CUMS +60.6972 mg·kg^{-1} Zuotai suspended in 2% starch solution), CUMS+Zuotai III (CUMS +606.972 mg·kg^{-1} Zuotai suspended in 2% starch solution), CUMS+IMI (CUMS +15 mg·kg^{-1} imipramine dissolved in 2% starch solution). During the period of CUMS paradigm, the experimental animals were correspondingly treated (intragastrical administration [ig],) with Zuotai or a positive drug (imipramine) or 2% starch solution every day for 42 days (ie, 6 weeks). The weight of the mice in each group was measured every week, and the volume of drugs was adjusted according to their weight. Treatment details are shown in Figure 1.

The drug equivalent dose conversion ratio of mouse versus human is 9.1, and was adopted in this study. The clinical dose of Zuotai is 0.667 mg·kg^{-1}; therefore, the equivalent dose in a mouse is 6.06972 mg·kg^{-1}. The experiments were conducted with 1, 10, and 100 times the clinical equivalent doses of Zuotai in mice to explore the dose–effect relationship.

Sucrose preference test

The SPT is an important method that is mainly used to evaluate anhedonia, which is the core symptom of depression. The SPT was conducted in this study as described elsewhere.[37] Thus, 72 h before the test, the mice were allowed to adapt to drinking sugar water. Briefly, two water bottles were simultaneously placed in each cage; one bottle was filled with 1% sugar solution, whereas the other contained pure water. The positions of the two bottles were switched every 12 h. Then, the SPT was conducted at 7:00 pm on days 0 and 42 of the present study. The mice were housed in individual cages, and were free to access either of the two bottles containing 1% sucrose solution or water. The volumes of consumed sucrose solution and water were recorded after 2 h. The sucrose preference ratio (SPR) was calculated according to the following equation: SPR = Sucrose intake (g)/[Sucrose intake (g) + Water intake (g)] ×100%.

Forced swimming test

The FST was used to evaluate the learned helplessness state of depressed mice on Day 44 of the experiment. The method was conducted as described by Porsolt et al.[35] Briefly, 60 min prior to the FST, mice were moved to a quiet test room to reduce their tension. Then, the mice were individually placed in a glass cylindrical container (total volume: 1,000 mL, 21 cm in height and 12 cm in diameter) that was filled with water (23°C±2°C) to a depth of 10 cm. Each mouse was exposed to a test session for 6 min, and the whole experimental process was recorded by a high-definition digital video camera. The duration of immobility was accurately scored during the last 4 min of the total swimming time by using a timer, which was handled by a blinded observer. A mouse was judged to be in an immobile posture when it remained in a passively floating state in water without struggling or was swimming just to keep its head above the water.

Tail suspension test

The TST is another important method that is used to evaluate the learned helplessness state of the depressed mice, similarly as a forced swimming test. On Day 45 of the experiment, the TST was conducted using a method adapted from Steru et al,[34] with a minor modification. Briefly, 60 min prior to the experiment, the mice were moved to a quiet test room to reduce tension. Acoustically and visually isolated mice were suspended by their tail from a ledge with adhesive tape (5 cm in width), 10 cm above the tabletop, for 6 min. The tape was placed approximately 3 cm from the tip of the tail. Immobility was defined as the absence of movement, with the time of immobility recorded during the last 4 min of the

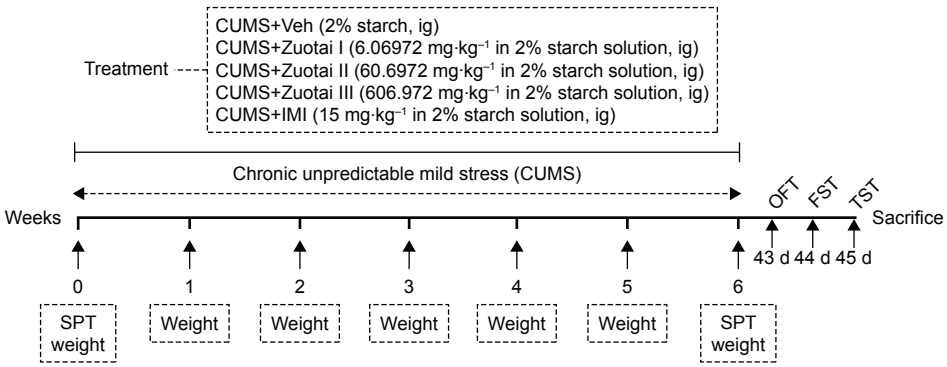


Figure 1 The experimental design.
Abbreviations: CUMS, chronic unpredictable mild stress; ig, intragastrical administration; IMI, imipramine; Veh, vehicle; SPT, sucrose preference test; FST, forced swimming test; TST, tail suspension test; OFT, open field test.

total suspended time by using a timer, which was handled by an observer blinded to the drug treatment.

Open field test

The OFT is often used in animal models of anxiety and depression. It is not usually thought of as a model of "depression-like" behavior so much as "anxiety-like" behavior. It is appropriate to use in this case, however, because many antidepressants also have anti-anxiety behaviors. The OFT was conducted according to the method described in a previous report[38] on Day 43 of the present experiment. Sixty minutes before the OFT, the mice were moved to the test room and allowed to adapt to the experimental environment. The mice were placed in the center of an OFT box (500×500×415 mm), and the curtain of this experimental apparatus was rapidly pulled. The nine-rectangle-grid mode was selected, and the ratio of the central area versus the quadrilateral area was 0.5. Then, spontaneous locomotor activities (total immobile time, total movement distance, and percentage of time to cross the center) of each mouse were measured during a 5-min period by using this apparatus (OFT-100; Chengdu Techman Software Co., Ltd., People's Republic of China).

Neuroendocrine hormones in the HPA axis

At 9:00–10:00 am on Day 46 of the experiment, blood was collected from the posterior orbital venous plexus of each mouse without anesthesia (then, the mouse was immediately sacrificed by cervical dislocation) and placed into a coagulation tube, kept for 1 h at room temperature, and the serum was separated by centrifugation at 4,000 rpm for 10 min at 4°C. Then, serum Cort and ACTH levels were determined according to the methods provided in the immunoassay kits. Thereafter, the hypothalamuses were isolated from the brain and placed on ice. Each hypothalamus was placed into 1.5 mL Eppendorf tubes, to which phosphate-buffered solution (pH 1.0–2.0) was added at 9 times the volume of the hypothalamus. Each hypothalamus was homogenized using a tissue homogenizer (frequency: 60 Hz, speed: 1,800 rpm) for 2 min and then centrifuged at 1,000 *g* for 20 min, and the supernatant was collected. Finally, the CRH level was determined according to the instructions provided in the ELISA kit.

Monoamine neurotransmitters

The levels of the monoamine neurotransmitters (5-HT and NE) in the serum collected from the mouse were determined according to the protocol provided in the ELISA kits (Cloud-Clone Corp.).

Total mercury in hypothalamus and hippocampus

The hypothalamus and hippocampus of the subjected mice were collected on Day 46 of the experiment, and used in the determination of mercury levels using an automatic direct mercury analyzer (DMA-80; Milestone Co., Ltd., Italy).

Statistical analysis

All data were expressed as the mean ± SEM of each group. The multiple comparisons of data were analyzed using One-way ANOVA Post Hoc Multiple Comparisons with the Duncan method (at least three conditions). The independent samples *t*-test was used for pairwise comparisons. For the 6 weeks data of mice body weight, two-way ANOVA with repeated measures was used (two factors: dose and time). Statistical analysis was done and graphs were constructed using SPSS 20.0 and Office 2013. A *P*-value of <0.05 was considered statically significant.

Results

Body weight

Depression is often accompanied by significant body weight loss or gain. The body weight of mice in each group were monitored weekly during all the studies (Figure 2A). No differences in baseline body weight were observed among each group (at Week 0: $P>0.05$). In the second week of the CUMS stress procedure, the body weight of mice in the CUMS+Veh group ($P<0.001$), CUMS+Zuotai I group ($P<0.05$), CUMS+Zuotai II group ($P<0.001$), CUMS+Zuotai III group ($P<0.001$), and CUMS+IMI group ($P<0.001$) significantly decreased, compared to the age-matched control group. After 6 weeks continuous treatment with Zuotai (6.06972 and 60.6972 mg·kg^{-1}) or imipramine (15 mg·kg^{-1}), weight loss in the CMUS mice was significantly improved compared to that in the CMUS+Veh group ($P<0.001$ for CUMS+Zuotai I group, $P<0.05$ for CUMS+Zuotai II and CUMS+IMI groups). The absolute change in mice body weight (Δweight = Final body weight – Initial body weight) is shown in Figure 2B. The body weight decreased significantly in mice after 6 weeks of CUMS exposure compared to that of the control group ($P<0.001$), which was significantly improved by Zuotai (6.06972, 60.6972, and 606.972 mg·kg^{-1}) or imipramine (15 mg·kg^{-1}).

Sucrose preference test

As a main index for evaluating anhedonia, the SPR of each group of mice was measured at Week 0 and 6 weeks post CUMS and drug procedures (Figure 3). The results showed that there was no significant difference between groups

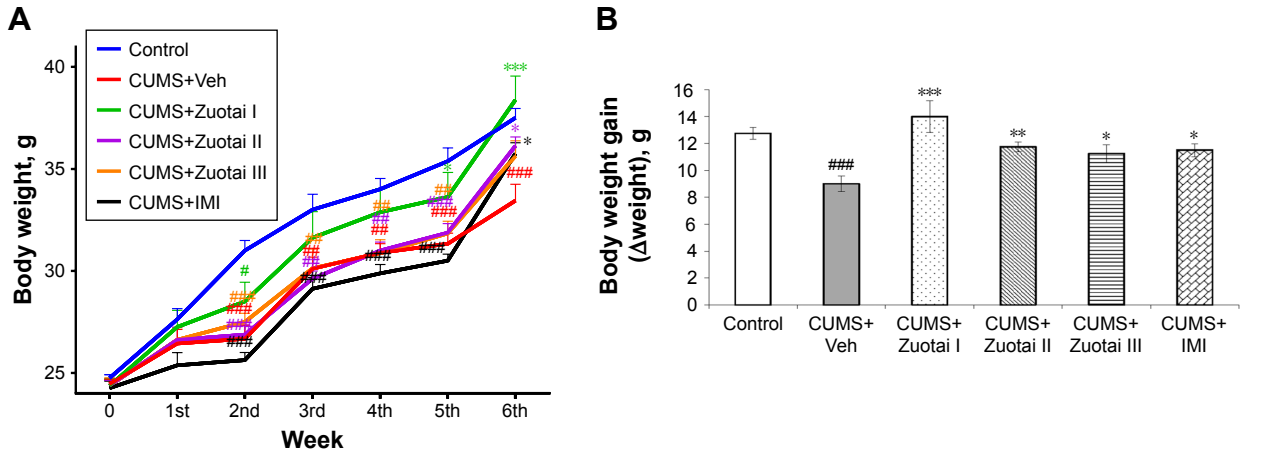


Figure 2 Changes in body weight in mice over the course of 6 weeks. (**A**) The changing trend of body weight during 6 weeks of treatment (mean ± SEM, n=10) compared to the control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$; compared to the CUMS+Veh group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (**B**) Body weight gain (Δweight) of mice after 6 weeks treatment (mean ± SEM, n=10). CUMS+Veh vs control, $^{\#\#\#}P<0.001$; compared to the CUMS+Veh group, $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.
Abbreviations: CUMS, chronic unpredictable mild stress; IMI, imipramine; Veh, vehicle.

in baseline SPR scores (Week 0: $P>0.05$, Figure 3A). After 6 weeks of the CUMS procedure, the SPR of the CUMS+Veh group mice was significantly decreased (Week 6: $P<0.001$, Figure 3B) in comparison to that of the control group. However, compared with the CUMS+Veh group, the SPR of the mice treated with Zuotai or imipramine increased significantly (Week 6: $P<0.01$, $P<0.001$, respectively; Figure 3B).

Forced swimming test

After a 6-week CUMS procedure, the CUMS-treated mice exhibited a pronounced increase in the duration of immobility, compared to the non-CUMS mice ($P<0.01$). Zuotai significantly attenuated the duration of immobility in CUMS mice ($P<0.05$ for 6.0697 mg·kg^{-1} group and 60.697 mg·kg^{-1} group, and $P<0.01$ for 606.97 mg·kg^{-1} group) compared to the CUMS+Veh group. In addition, treatment with imipramine (15 mg·kg^{-1}) resulted in a significant reversal in the CUMS-induced increase in the duration of immobility ($P<0.05$ vs CUMS+Veh group) in CUMS mice (Figure 4).

Tail suspension test

Results from the TST of each group are shown as in Figure 5. Six-week CUMS treatment significantly increased the immobility time of Kunming mice ($P<0.01$). Zuotai (6.06972, 60.6972, and 606.972 mg·kg^{-1}) or imipramine (15 mg·kg^{-1}) remarkably attenuated CUMS-induced immobility time compared to the mice in the CUMS+Veh group ($P<0.001$ or $P<0.05$, Figure 5).

Open field test

To eliminate nonspecific influence, we determined spontaneous locomotor activity (total movement distance, total immobility time, and the percentage of time to cross the center) in all mice using the OFT before the FST and the TST. The OFT results are shown in Figure 6A–D. After 6 weeks of the CUMS procedure, the total movement distance and the percentage of time to reach the central area significantly decreased, whereas total immobility time increased significantly compared to that of control mice. Furthermore, spontaneous locomotor activities of CUMS

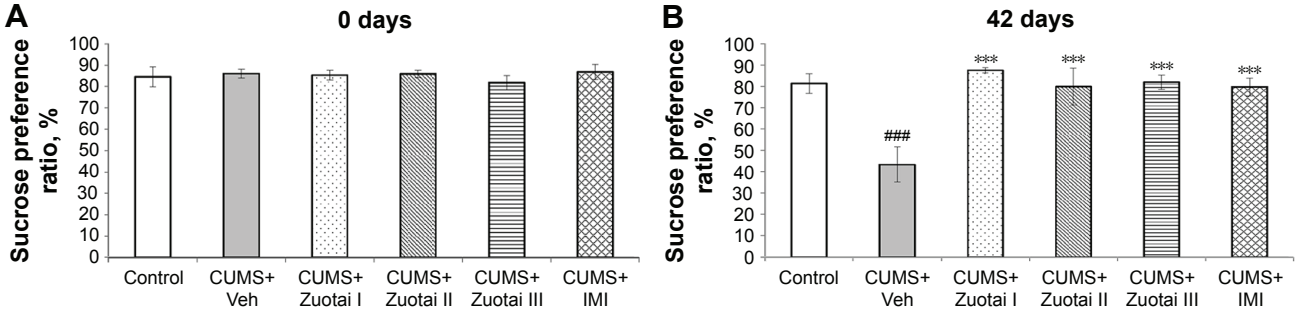


Figure 3 Sucrose preference ratio scores of each group of mice at days 0 and 42 of administration. (**A**) Baseline SPR scores of each group of mice at Day 0 of administration (mean ± SEM, n=10), compared to control group. (**B**) Sucrose preference ratio of each group after 6 weeks of treatment (mean ± SEM, n=10), CUMS+Veh compared to control group, $^{\#\#\#}P<0.001$; compared to CUMS+Veh group, $^{***}P<0.001$.
Abbreviations: CUMS, chronic unpredictable mild stress; IMI, imipramine; Veh, vehicle.

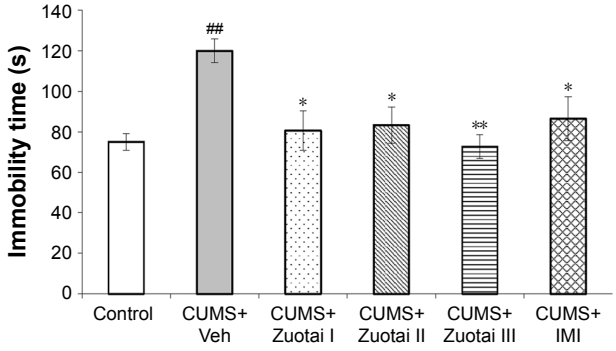


Figure 4 Duration of immobility in mice in the forced swimming test (mean ± SEM, n=10). CUMS+Veh group compared to control group, $^{\#\#}P<0.01$; compared to CUMS+Veh group, $^{*}P<0.05$, $^{**}P<0.01$.
Abbreviations: CUMS, chronic unpredictable mild stress; IMI, imipramine; Veh, vehicle.

mice were ameliorated by Zuotai (6.06972, 60.6972, and 606.972 mg·kg^{-1}) or imipramine (15 mg·kg^{-1}).

Neuroendocrine hormones in the HPA axis

Levels of three core neuroendocrine hormones (CRH, ACTH, and Cort) in the HPA axis were determined and are shown in Figure 7A–C. The CRH level in the hypothalamus and levels of serum ACTH and Cort were significantly upregulated after 6 weeks of continuous CUMS treatment, and could be reversed by Zuotai (6.06972 and 60.6972 mg·kg^{-1}, $P<0.05$ or $P<0.01$) or imipramine (15 mg·kg^{-1}, $P<0.05$ or $P<0.01$) treatment. However, Zuotai failed to show any significant changes in levels of CRH, ACTH and Cort at a dose of 606.972 mg·kg^{-1}, compared to that in the CUMS+Veh group.

Monoamine neurotransmitters (NE and 5-HT) in serum

Given that monoamine neurotransmitters (NE and 5-HT) play critical roles in emotional and cognitive functions and in the development of the nervous system, their serum levels were determined and are shown in Figure 8A and B. After 6 weeks of CUMS administration, serum 5-HT and NE levels were significantly downregulated. Meanwhile, the serum NE level was remarkably rescued by Zuotai (6.06972, 60.6972, and 606.972 mg·kg^{-1}) or imipramine (15 mg·kg^{-1}) after 6 weeks of oral administration (Figure 8A). However, the level of serum 5-HT was only reversed by Zuotai at the doses 6.06972 and 60.6972 mg·kg^{-1}, but not at a dose of 606.972 mg·kg^{-1} (Figure 8B).

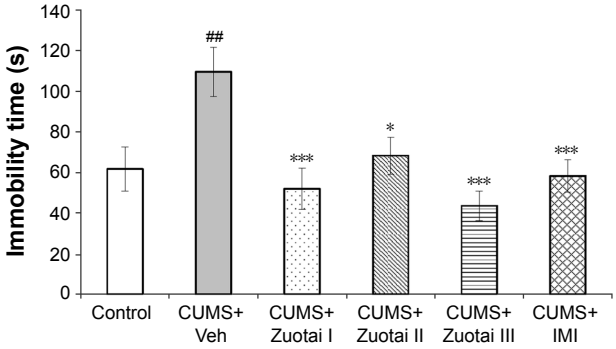


Figure 5 Immobility time of each group of mice in the tail suspension test (mean ± SEM, n=10). CUMS+Veh group compared to control group, $^{\#\#}P<0.01$; compared to CUMS+Veh group, $^{*}P<0.05$, $^{***}P<0.01$.
Abbreviations: CUMS, chronic unpredictable mild stress; IMI, imipramine; Veh, vehicle.

Mercury levels in the hypothalamus and hippocampus

The total mercury concentrations in the hypothalamus and hippocampus of each group mice were detected and are shown in Figure 9. No significant change in mercury level was observed in the hypothalamus and hippocampus in CUMS+Veh and CUMS+IMI mice compared to control mice. After 6 weeks of continuous Zuotai treatment (6.06972, 60.6972, and 606.972 mg·kg^{-1}), the mercury level in the hypothalamus of mice was significantly increased in a dose-dependent manner. Moreover, the mercury level in the hippocampus of mice treated with Zuotai (60.6972 and 606.972 mg·kg^{-1}) was significantly higher than that of the control group, and also changed in a dose-dependent manner.

Discussion

The present study has elucidated the antidepressant activity of Zuotai and its potential mechanism. Zuotai significantly ameliorated depressive behaviors in CUMS-treated mice, characterized by increased SPR in the SPT, increased total movement distance and percentage of time to cross the center in the OFT, and improved body weight as well as motor activities in FST, TST, and OFT. Moreover, Zuotai suppressed the increase in the levels of three important neuroendocrine hormones (CRH, ACTH, and Cort) on HPA axis pathway, and rescued the decrease in the levels of the monoamine neurotransmitters (NE and 5-HT) in CUMS-treated mice. Further, we found that Zuotai could dose-dependently increase the level of mercury in the hypothalamus and hippocampus of mice.

CUMS mouse model was successfully established

The CUMS model is a classic model for testing the efficacy of antidepressants. In the procedure for establishing the CUMS model, rodents are chronically exposed to multiple stress factors that are unpredictable and pose no threat to their survival; this procedure is consistent with the etiology of human depressive disorders.[30,31] The CUMS model not

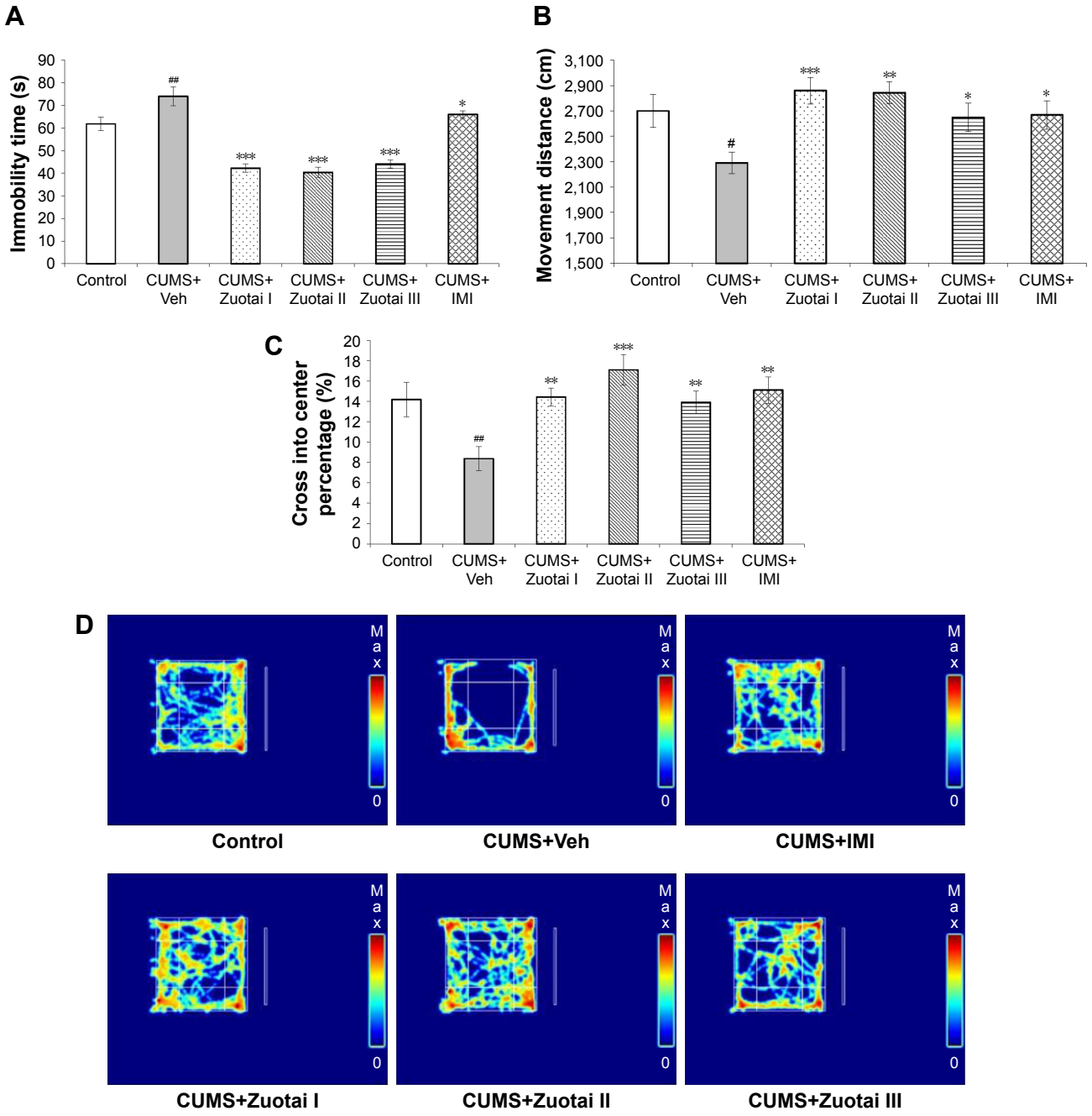


Figure 6 The OFT results of each group mice. (**A**) Total immobility time of each group mice in OFT (mean ± SEM, n=10), CUMS+Veh group compared to control group, $^{\#\#}P<0.01$; Compared to CUMS+Veh group, $^{*}P<0.05$, $^{***}P<0.001$. (**B**) Total movement distance of each group mice in OFT (mean ± SEM, n=10), CUMS+Veh group compared to control group, $^{\#}P<0.05$; compared to CUMS+Veh group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (**C**) Percentage of time for each group mice to reach the central area in OFT (mean ± SEM, n=10), CUMS+Veh group compared to control group, $^{\#\#}P<0.01$; compared to the CUMS+Veh group, $^{**}P<0.01$, $^{***}P<0.001$. (**D**) The motion trajectory map of each group of mice in the OFT (red refers to the maximum rest time of mouse in the region, and blue refers to no mouse resting in the region).
Abbreviations: CUMS, chronic unpredictable mild stress; IMI, imipramine; OFT, open field test; Veh, vehicle.

only simulates anhedonia – a core symptom of some human depressions – but also recapitulates other major depressive symptoms such as anxiety-like and depressive-like moodiness, decline in sociability and spontaneous locomotor functions, abandonment of violation and attack ability, sex apathy, and weight loss.[30,32] In the present study, our CUMS mice displayed conspicuous depressive-like symptoms such as anhedonia, decline in spontaneous locomotor functions, enhanced learned helplessness state, and weight loss, which is consistent with findings in previous reports. This indicates that we successfully established a CUMS mouse model.

Zuotai ameliorates depressive-like behaviors of CUMS mice

Anhedonia, which is a core symptom of depressive disorders, refers to broadly retarded reactions toward rewards or the

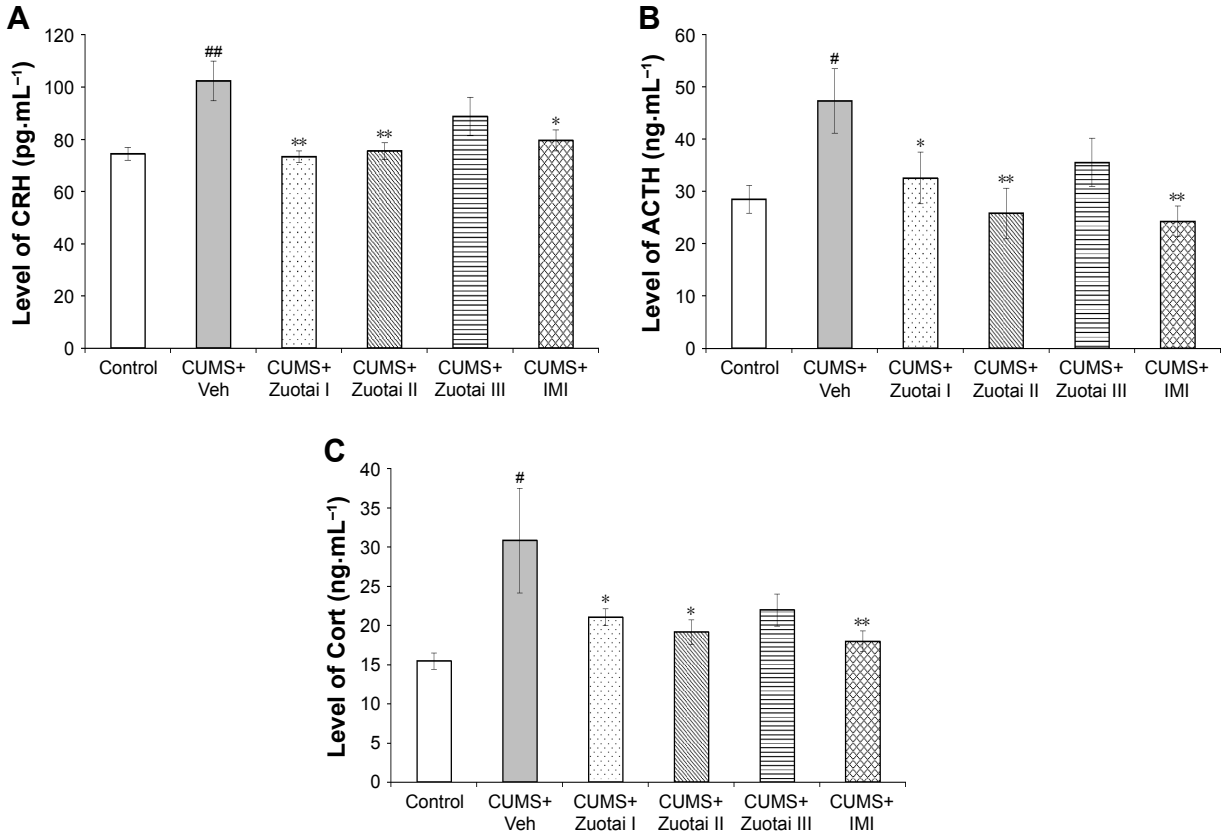


Figure 7 Neuroendocrine hormones (CRH, ACTH, and Cort) in the HPA axis. (**A**) CRH level of each group mice in the hypothalamus (mean ± SEM, n=10); (**B**) Serum ACTH levels of each group of mice (mean ± SEM, n=10); (**C**) Serum Cort levels of each group of mice (mean ± SEM, n=10). CUMS+Veh group compared to the control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; compared to the CUMS+Veh group, $^{*}P<0.05$, $^{**}P<0.01$.

Abbreviations: ACTH, adrenocorticotropic hormone; Cort, corticosterone; CRH, corticotropin-releasing hormone; CUMS, chronic unpredictable mild stress; IMI, imipramine; Veh, vehicle.

inability to enjoy happiness.[31,39] Previous reports had shown that anhedonia in the form of reduction in SPR in mice is significantly reversed by antidepressants such as imipramine and fluoxetine.[40,41] In the current study, the administration of Zuotai remarkably improved the SPR in the CUMS mice, thereby indicating that Zuotai may have potential effects on amelioration of the core symptoms of depression. Moreover, our results showed that Zuotai could reverse weight loss in CUMS mice. Major depressive disorder (MDD) is often associated with appetite changes (loss of appetite or increased appetite) and subsequent weight changes,[4,5,42] and treatment with antidepressants is often associated with weight gain in

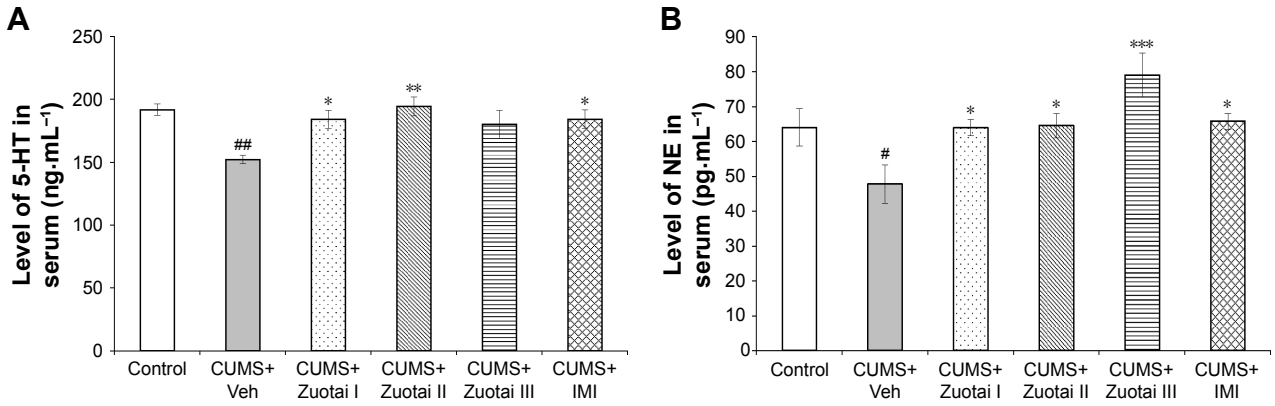


Figure 8 Serum levels of monoamine neurotransmitters (NE and 5-HT). (**A**) Serum 5-HT levels of each group mice after 6 weeks of administration (mean ± SEM, n=10); (**B**) Serum NE levels of each group mice after 6 weeks of administration (mean ± SEM, n=10), CUMS+Veh group compared to control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; compared to the CUMS+Veh group, $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

Abbreviations: CUMS, chronic unpredictable mild stress; 5-HT, 5-hydroxytryptamine; IMI, imipramine; NE, norepinephrine; Veh, vehicle.

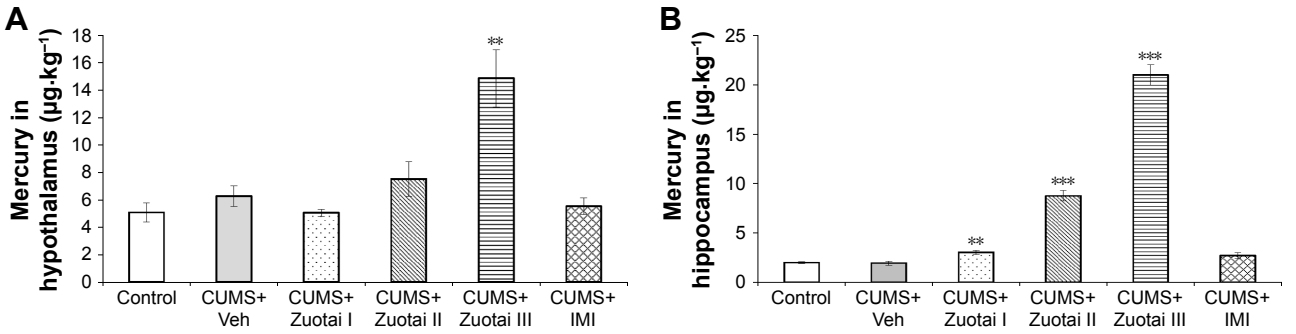


Figure 9 Mercury levels in both cerebric areas (hypothalamus and hippocampus) of each group of mice. (**A**) Mercury levels in the hypothalamus of each group of mice after 6 weeks of drug administration (mean ± SEM, n=10); (**B**) Mercury levels in the hippocampus of each group mice after 6 weeks of treatment administration (mean ± SEM, n=10). Compared to control group, $^{**}P<0.01$, $^{***}P<0.001$.
Abbreviations: CUMS, chronic unpredictable mild stress; IMI, imipramine; Veh, vehicle.

patients with for depression.[4,42] Learned helplessness is an important indicator for assessing depressive-like behavior. TST and FST are two classical methods for evaluating the learned helplessness state of rodents, and in screening antidepressants.[34,35] These two methods have nearly the same design principle – that is, preventing animals from escaping a harsh environment, thereby leading to immobility behavior.[43,44] When mice are suspended by their tail or forced to swim in water, they are subjected to short-term inescapable stress and they adopt an immobile posture.[43] However, when antidepressants are administered prior to the test, mice will actively pursue escape-directed behaviors over longer periods of time.[43,44] These two methods are sensitive for the screening of antidepressant drugs – for example, imipramine, fluoxetine, and other antidepressants significantly reduced the immobile posture during TST and FST in depressed mice.[43,45,46] In the present study, the application of Zuotai (6.0697, 60.697, and 606.97 mg·kg^{-1}) resulted in a significant reduction in the immobile posture during both TST and FST in CUMS mice, and this was similar to the change with the positive drug imipramine.

The OFT is a generally accepted and straightforward test for investigating depressive-like, anxiety-related, and exploratory behavior of rodents.[47] Chronic stress can lead to depressive-like and anxiety-like behaviors in mice that are expressed in terms of a reduction in spontaneous activity such as an increase in rest time, a decrease in movement distance and time of crossing into the center, and a reduction in standing times.[48] Our results showed that the CUMS procedure significantly reduced the total distance of movement and the time of crossing into the center, and also reduced the resting time of normal mice, whereas these behaviors could prominently be reversed by the administration of Zuotai or imipramine.

Zuotai regulates the HPA-axis pathway and monoamine neurotransmitters in CUMS mice

The occurrence of depression is closely related to the lack/decrease of monoamine neurotransmitters, which is involved in HPA-axis hyperactivity.[26,28,49] Under long-term chronic stress and social stress, HPA-axis hyperfunction can be induced, which in turn further leads to a reduction in monoamine neurotransmitters in the central and peripheral nervous systems, thereby resulting in depressive-like symptoms.[26] Antidepressant treatment relieves HPA-axis hyperactivity and improves levels of monoamine neurotransmitter.[50,51] In the present study, we found that Zuotai could significantly reverse HPA-axis hyperactivity at doses of 6.0697 and 60.697 mg·kg^{-1} by reducing serum CRH, ACTH, and Cort levels. Furthermore, we observed that Zuotai could remarkably upregulate the level of monoamine neurotransmitters (5-HT and NE). Therefore, the antidepressant effect of Zuotai on CUMS mice may involve inhibition of the HPA axis and cause upregulation of monoamine neurotransmitters levels. However, Zuotai failed to attenuate the hyperactivity of the HPA axis at a higher dose (606.97 mg·kg^{-1}), which implies that the inhibitory effect of Zuotai on HPA-axis hyperactivity involves a certain threshold, and higher doses may be ineffective.

Potential risk of mercury from Zuotai on the nervous system

As a classical processing mixture containing mercury in Tibetan medicine, Zuotai continues to be used in compound preparations for the treatment of various diseases. Mercury is an important toxic heavy metal element that is ubiquitous in the environment, and its toxicity depends on its chemical forms. The mercury chemical species in Zuotai is insoluble

cubic crystal mercuric sulfide (β-HgS, K_{sp} is 1.6×10^{-52}),[52] and its toxicity is extremely low.[53] Our previous study showed that chronic administration of Zuotai to mice resulted in the accumulation of mercury in the kidneys and liver, whereas only trace amounts were detected in the brain.[53] However, the extent of mercury accumulation in different encephalic regions treated by Zuotai remains unclear. In the present study, some level of mercury deposition was observed in the hypothalamus and hippocampus in CUMS mice treated with Zuotai in a dose-dependent manner. Therefore, we suspect that it may pose a potential risk for the use of Zuotai in the clinic. Meanwhile, Zuotai could ameliorate depressive-like behaviors in CUMS mice. Here, we made a bold speculation that the protective effect of Zuotai on depression may be associated with the deposition of mercury from Zuotai in brain.

Probable relationship of mercury deposition and HPA-axis function

Mercury is a well-known endocrine disruptor in the environment. However, mercury levels in vivo and its function on the HPA axis remain elusive, to date. Previous studies have shown that the concentration of Cort, which is one of the key signaling molecules in the HPA-axis pathway, is negatively correlated with mercury levels in the body.[54–56] However, some other studies have reported that ACTH and CRH – important signaling molecules in the HPA axis – were positively correlated with mercury levels in vivo.[57,58] It has also been reported that the level of Cort in the HPA axis is not associated with mercury levels in vivo.[59,60] Therefore, it is difficult to draw a general picture of mercury–HPA-axis relationships at this point.

Conclusion

In summary, the present study has found that Zuotai can ameliorate depression-like behaviors in CUMS mice, and revealed the underlying mechanism that involves the inhibition of the HPA axis neuroendocrine pathway, which can further upregulate the level of monoamine neurotransmitters and finally play a role to ameliorate depressive mood. However, Zuotai can cause significant mercury deposition in the hypothalamus and hippocampus at high doses and with long-term administration, which may pose a potential risk to the central nervous system. Therefore, mechanisms to balance the risk and the efficacy of Zuotai need to be further studied in future research. Taken together, these findings provide new insights into the interventional effect of Zuotai on depression and its potential risk necessitating mercury monitoring.

Abbreviations

HPA, hypothalamic–pituitary–adrenal; CUMS, chronic unpredictable mild stress; IMI, imipramine; SPT, sucrose preference test; SPR, sucrose preference ratio; FST, forced swimming test; TST, tail suspension test; OFT, open field test; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; Cort, corticosterone; 5-HT, 5-hydroxytryptamine or serotonin; NE, norepinephrine; ELISA, enzyme-linked immunosorbent assay.

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Disclosure

The authors report no conflicts of interest in this work.

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