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Evaluating the anti-depression effect of the Xiaoyaosan prescription on chronically-stressed mice

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Dear Editor:

We are pleased to submit this paper entitled "Compound Prescription Xiaoyaosan Improves Depressive-Like Behaviors of Chronically Stressed Mice" by Zhi-Yi Yan and Xiao-Juan Li for publication as a protocol in *JoVE*.

In this report, we show that Xiaoyaosan, a Chinese herbal formula that has widely used in the treatment of mental disorders, the intervention of Xiaoyaosan has a good effect on the overall improvement of patients with dpression. The current study provides evidence supporting the efficacy of Xiaoyaosan for treating depressive-like behaviors and contributes to a further understanding of the important role of Xiaoyaosan in ameliorating depression. We believe that this manuscript is appropriate for publication in *JoVE*, because our protocol presents evidence supporting the validity and reliability of complementary and alternative medicine in the treatment of depressive-like behaviors using a traditional Chinese herbal formula.

We hereby declare that we have no potential competing interests. All authors have read and approved the final version of the manuscript. This manuscript has not been published and is not under consideration for publication elsewhere.

Thank you for your consideration, and we look forward to hearing from you at your earliest convenience.

Sincerely,

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TITLE:

Evaluating the Anti-Depression Effect of Xiaoyaosan on Chronically-stressed Mice

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KEYWORDS:

Xiaoyaosan, Major depressive disorder, Chronic unpredictable mild stress, Depression, Behavior, Open field test, Sucrose preference test

SUMMARY:

Here, we present a protocol to establish the mouse depressive model, observe the behavior changes associated with chronic unpredictable mild stress (CUMS), and evaluate the anti-depression effect of Xiaoyaosan.

ABSTRACT:

In addition to the standardized use of antidepressant medications and psychotherapy, the usage of traditional Chinese medicine has lead to an overall improvement of patients with major depressive disorder (MDD). Therefore, the purpose of this study was to establish the mouse depressive model, observe the behavior changes associated with chronic unpredictable mild stress (CUMS), and then evaluate the anti-depression effect of Xiaoyaosan. Mice were randomly divided into four groups: a control group, a model group, a treatment group with Xiaoyaosan, and a treatment group with fluoxetine. All mice were individually kept in cages, and depression was induced in the mice by exposing them to several designed manipulations of CUMS for 21 days, as described in the protocol. Mice in the control group and model group received 0.5 mL of distilled

water, while mice in the treatment groups received either Xiaoyaosan (0.25 g/kg/d) or fluoxetine (2.6 mg/kg/d). The drugs used in the study were given intragastrically daily during the entire three weeks. To estimate the depressive-like behaviors, a series of parameters including the coat state, body weight, open field test score, and sucrose preference test score were recorded. Data analysis showed that behaviors of model mice were significantly changed compared to behaviors of mice in the control group, which were improved by the treatment of Xiaoyaosan and fluoxetine. The current findings demonstrated the anti-depression effects of Xiaoyaosan on the behaviors of CUMS-induced mice and revealed that compounds from the Xiaoyaosan prescription may be worthwhile for treating depression, considering their beneficial effects on depressive-like behaviors.

INTRODUCTION:

MDD is a chronic, highly recurrent mental disease that presents with persistent low mood, depression, low self-esteem, mental retardation, loss of interest, sleep disorders, profoundly pessimistic or suicidal tendencies; it has caused great harm to human health and social stabilization[1]. It has become one of the world's primary cause of disability, and it may be one of the main leading causes of disease burden by 2030 according to the World Health Organization[2,3]. Currently, antidepressant medications and psychotherapy are considered to be the first choice to treat MDD. Nevertheless, an enormous number of patients diagnosed with MDD do not respond to continuous medication or therapy, and they are listed as having treatment-resistant depression[4].

Xiaoyaosan is a classic Chinese compound[5] that has been widely used to treat psychological diseases for thousands of years since the Song dynasty in China[6]. It is composed of 8 Chinese herbs: *Radix Bupleuri*, *Radix Paeoniae Alba*, *Radix Angelicae Sinensis*, *Poria*, *Rhizoma Atractylodis Macrocephalae*, *Radix Glycyrrhizae*, *Rhizoma Zingiberis Recens*, and *Herba Menthae*. Xiaoyaosan contains multiple constituents, has multiple targets, and utilizes multiple pathways[7]. Xiaoyaosan has been commonly shown to be a safe and effective formula for treating various diseases related to mental disorders, and modern studies have also focused on the anti-depression mechanism of this compound[8-10]. The pathogenesis of MDD is yet unclear, even though there are multifarious hypotheses, including the monoamine hypothesis, the neurotransmitter receptor hypothesis, the neuroendocrine function change hypothesis, the immune system abnormality hypothesis, *etc*. Several studies have shown that the antidepressant effect of Xiaoyaosan has a connection with the material bases of the above hypotheses[11,12], and its therapeutic action manifests as an improvement to depressive-like behaviors[13].

Moreover, with the prevalence of depression, modern society has suffered from reductions in the work force, early retirements, and expensive treatments[14]. Expensive treatment needs strict medical attention, which means a range of increased medical costs and precise anticipation of precise undesirable outcomes as well as side effects[15]. The Chinese herb Xiaoyaosan has accessible components, unique effects, and slight side effects. Its application can play a role in

reducing the burden of modern medical treatment. Therefore, to provide a more theoretical basis for the application of Xiaoyaosan, an alternative clinical therapy should be offered for the treatment of depression, the incidence of which increases year by year, and should improve human health at the same time. This paper describes a protocol involving the use of Xiaoyaosan to prevent the depressive-like behaviors by establishing the depressive model of the mouse. The open field test and sucrose preference test were used to verify the antidepressant effects of Xiaoyaosan.

PROTOCOL:

This protocol was approved by the Animal Ethics Committee of Beijing University of Chinese Medicine and was in accordance with all guidelines for the Care and Use of Laboratory Animals of China.

1. Preparing Drugs for the Experiment

Note: The Chinese herbs were purchased and then authenticated by Dr. B. Liu of the Beijing University of Chinese Medicine. Xiaoyaosan contains the following eight traditional Chinese drugs in a ratio of 6:6:6:6:3:6:2:2: Radix Angelicae Sinensis, Radix Paeoniae Alba, Poria, Radix Bupleuri, Radix Glycyrrhizae, Rhizoma Atractylodis Macrocephalae, Herba Menthae and Rhizoma Zingiberis Recens,.

1.1. Prepare Xiaoyaosan as previously described[16].

Note: We extracted these compounds in the Chinese medicine preparation room of the China-Japan Friendship Hospital, and the extraction yield was 18.8%.

1.2. Verify the quality of Xiaoyaosan by high-performance liquid chromatography-mass spectrometry analysis (LC-MS/MS)[17].

2. Preparing Mice for the Experimental Procedure

Note: For the experimental procedure, 60 healthy male C57BL/6J mice aged 12 weeks were selected, with each mouse weighing between 18 and 22 g.

2.1. Keep mice individually in cages in a typical Specific Pathogen Free (SPF) laboratory animal room (22 ± 1 °C, 12 h/12 h dark/light cycle, relative humidity: 30%–40%) for one week before the CUMS procedure.

2.2. Randomly divide all mice into a control group, a model group, a Xiaoyaosan treatment group, or a fluoxetine treatment group according to their body weight. Each group has 15 mice.

2.3. Before initiation of the experiment, number the mice in each group on their tail using a marking pen.

2.4. Test all mice using a five-day sucrose preference test (refer to step 5) and an open field test (refer to step 6).

3. Induction of Depression in Mice

Note: To induce depression, the mice in the model group and two treatment groups underwent CUMS for 21 days[18]. The plan can be changed according to the practical situation, but the principle that the same stressor cannot be used continuously should be followed.

3.1. Expose mice daily to any two of the following seven stressors for 21 consecutive days[19]. Our modeling plan is designed as follows:

3.1.1. On Monday, expose mice to food deprivation (empty the feed of each cage for 24 h, 8:00 a.m. to 8:00 a.m.) and water deprivation (remove the drinking bottle of each cage for 24 h, 8:00 a.m. to 8:00 a.m.).

3.1.2. On Tuesday, expose mice to restraint stress (restrain mice in a small plastic tub for 3 h, 8:00 a.m. to 11:00 a.m.) and empty cages (remove the padding in each cage for 11 h, 8:00 a.m. to 19:00 p.m.).

3.1.3. On Wednesday, expose mice to wet and soiled cages (pour water into each cage and keep wet for 24 h, 8:00 a.m. to 8:00 a.m.) and crowded cages (put five mice in one cage for 24 h, 8:00 a.m. to 8:00 a.m.).

3.1.4. On Thursday, expose mice to restraint stress and ice-cold swimming (force each mouse to swim in a clear glass aquarium with ice water for 5 min).

3.1.5. On Friday, expose mice to wet and soiled cages and food deprivation.

3.1.6. On Saturday, expose mice to restraint stress and crowded cages.

3.1.7. On Sunday, expose mice to empty cages and ice-cold swimming.

3.2. Record the coat state and body weight weekly until the end of modeling (day 21). Calculate the total score of the coat state as the sum of scores from seven different body parts: head, neck, dorsal coat, ventral coat, forepaws, tail, and hind paws. A score of one indicates a well-groomed coat, and a score of zero indicates an unkempt coat[20].

3.3. Perform a five-day sucrose preference test (refer to step 5) and an open field test (refer to step 6) at the end of experiment.

4. Administration of Xiaoyaosan

Note: The dose of Xiaoyaosan (0.25 g/kg/d) was selected on the basis of its satisfactory efficacy as previously described[9].

4.1. During the experiment, use distilled water to prepare the Xiaoyaosan and fluoxetine suspensions, and make sure to use immediately.

4.2. Give 0.5 mL of distilled water to the mice in the control group and model group by intragastric administration every day before modeling. Meanwhile, give Xiaoyaosan (0.25 g/kg/d) or fluoxetine (2.6 mg/kg/d) to the mice in the two treatment groups.

5. Sucrose Preference Test

Note: No water or food deprivation was applied before the test. The protocol was performed as previously reported[21].

5.1. On the first two days, house the mice individually with two bottles filled with tap water.

5.2. On the next two days, give the mice two bottles filled with 1% sucrose solution.

5.3. On the last day, give the mice two bottles for 24 h, one with tap water and the other with 1% sucrose solution. To eliminate a side preference of the mice in drinking behavior, switch the position of the two bottles after 12 h.

5.4. Record the volume (mL) of both the consumed sucrose solution and tap water. Calculate the sucrose preference as follows: sucrose preference (%) = sucrose consumption/total liquid consumption × 100%.

6. Open Field Test

6.1. Perform the open field test in a dimly lit and quiet room with a four-sided wooden black lusterless box. Divide the floor into 25 equal squares by blue lines.

6.2. Clean the open-field box with 5% alcohol prior to the access of each mouse. Then, place the mouse in the center of the open field gently.

6.3. Suspend a high definition digital camera approximately 200 cm right above the field, and record the behavior of each mouse for 5 min.

6.4. Evaluate the mice behavioral video by the video-tracking system software and record the data of the total running distance and number of entries into the center zone.

6.5. Analyze the data of the total distance moved and number of entries into the center zone by statistical software.

REPRESENTATIVE RESULTS:

Note that the results have been previously published by Dr. Xiu-Fang Ding, *et al.*[18]

Data were expressed as the mean ± standard error of the mean (SEM). A one-way ANOVA or a non-parametric test was used for general data based on the normality test and the test for homogeneity of variance. The LSD test was used for the comparisons between groups. In addition, the repeated measurement process of the general linear model (GLM) was used to conduct one-way ANOVA analysis for repeated measured data (coat score and body weight), and a multivariate analysis process of variance was adopted to make comparisons between groups at each time point (LSD method).

The physical state of CUMS-induced mice

After exposing mice to three weeks of CUMS for induction of depression, a gradual dystrophy of coat state in the model group was induced; the dystrophy did not present after two weeks of stress, but it worsened at the end of the CUMS procedure (three weeks), and the coat score significantly decreased compared to that of the control group ($F(3, 56) = 25.08$, $p = 0.000$). The coat state of mice was notably improved after continuous treatment with Xiaoyaosan or fluoxetine compared to the model group ($F(3, 56) = 25.08$, both $p = 0.001$, **Figure 1a**). According to the weekly body weight data of each group, a significant reduction in body weight was manifested by the third week ($F(3, 56) = 16.149$, $p = 0.000$, **Figure 1b**), while Xiaoyaosan or fluoxetine treatment significantly increased the body weight compared with the model group ($F(3, 56) = 16.149$, both $p = 0.001$).

The sucrose preference test

Before stress exposure, mice in all groups had a similar sucrose preference (baseline condition, **Figure 2a**). However, a significant drop in sucrose preference was observed after three weeks of stress procedure in model group ($F(3, 56) = 41.379$, $p = 0.000$, **Figure 2b**). Xiaoyaosan and fluoxetine treatment effectively reversed these changes and significantly increased the sucrose preference compared with model group ($F(3, 56) = 41.379$, both $p = 0.000$).

The open field test

Before stress exposure, all mice exhibited a similar behavioral state (baseline conditions. **Figures**

2c and **2d**). However, after stress exposure for three weeks, the total distance moved in five minutes significantly differed between the groups. The total distance moved by mice in the model group was significantly lower ($F(3, 56) = 4.216$, $p = 0.003$, **Figure 2f**) than that of the control group. Furthermore, the number of entries into the central zone was also significantly different between groups. The number of entries into the central zone of the model group significantly decreased ($F(3, 56) = 2.809$, $p = 0.013$, **Figure 2e**) compared with the control group. Fluoxetine treatment prevented these changes in locomotion trend and significantly improved the total distance and the number of entries into the central zone ($F(3, 56) = 4.216$, $p = 0.029$; $F(3, 56) = 2.809$, $p = 0.004$). A similar phenomenon was also observed in the Xiaoyaosan treatment group ($F(3, 56) = 4.216$, $p = 0.014$; $F(3, 56) = 2.809$, $p = 0.029$).

FIGURE LEGENDS:

Figure 1: Effects of Xiaoyaosan on CUMS-induced changes in mice physical state. The mice coat state (a) and body weight (b) were measured weekly during the stress period. Data were expressed as the mean ± SEM, n = 15 per group. $^{**}p < 0.001$ versus control; $^{\Delta\Delta}p < 0.001$ versus the model. This figure has been modified from Ding, *et al.*[18]

Figure 2 Effects of Xiaoyaosan on CUMS-induced depressive-like behaviors. A battery of behavioral tests was conducted, and the following parameters were measured: sucrose preference (a, b), number of entries into the center zone (c, e), and the total distance moved (locomotor activity) (d, f). Data were expressed as the mean ± SEM, n = 15 per group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus control; $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, and $^{\Delta\Delta\Delta}p < 0.001$ versus model. This figure has been modified from Ding, *et al.*[18]

DISCUSSION:

The CUMS model used in this study is a common method to establish the stress diathesis model of depression[22]. Most manifestations of CUMS can be restored by antidepressant drugs, which indicated that this model method has a strong predictive validity[23]. By this method, animals receive chronic unpredictable stressful stimuli and develop core symptoms of major depression, including decreased place preference conditioning, anhedonia, and impaired emotion-like behaviors[24]. Here, in this protocol, the continuous usage of Xiaoyaosan or fluoxetine can improve the depressive-like behaviors of CUMS-induced model mice according to the results obtained in the experiment; the fluoxetine is as a positive control drug to prove the intervention effect of Xiaoyaosan.

The evaluation of coat score, unlike the open field test, forced swimming test, or novelty suppressed feeding test, is a measure that is not associated with depression in humans but is the most prevalent, reliable, and well-validated method for the depressive model of mouse[25]. In the present study, a gradual dystrophy of the coat state in the model group was induced and did not present after two weeks of stress but worsened at the end of the CUMS procedure (three weeks),

while the body weight was not significantly different among groups after one week of stress exposure. However, the difference of body weight in each group began to emerge after modeling for two weeks, thus leading to a subsequent decrease by the end of three weeks. The results indicate that these changes in physical state of CUMS-induced mice could be reversed by both Xiaoyaosan and fluoxetine treatment, suggesting that Xiaoyaosan could improve the physical states of depressed mice.

To further validate the CUMS model and verify the effect of the compound used in this protocol, a sucrose preference test and an open field test were used to assess the possible depressive behaviors in CUMS-induced mice. The reduction of anhedonia in sucrose preference was used to evaluate the depressive-like state in rodents, while the open field test, as an animal psychological test, is commonly used to evaluate the locomotor function and emotionality of rodents[26]. The result showed that 3 weeks of stress exposure had an obvious influence on the sucrose preference of mice, and the anhedonia was reversed by both the Xiaoyaosan and fluoxetine treatments. The open field test is commonly used to assess locomotor function and emotionality in laboratory animals[27]. In this study, it was used to analyze depressive behaviors. The results of the open field test showed that the total distance moved by the mice in the model group was significantly lower than that of the control group. Furthermore, the number of entries into the central zone by the model group of mice was significantly lower than that of the treatment group of mice, which suggested that CUMS-induced mice displayed depressive-like behaviors. The study also showed that Xiaoyaosan and fluoxetine could ameliorate the changes presented in the open field test.

In agreement with the similar results discussed in previous studies, this paper showed that CUMS causes a decrease in sucrose consumption and locomotor activity. The continuous usage of Xiaoyaosan was effective for the prevention and treatment of CUMS-induced depression in mice. Many studies have examined depression, an advanced understanding of an animal model could be the key to clarifying the mechanism of depression[28]. The CUMS method described in this protocol can effectively display the depression state of mice. Unlike the chronic mild stress (CMS) or chronic immobilization stress (CIS) method[29,30], the key point of CUMS lies in the selection of unpredictable stressors and the rational application of model evaluation. To further confirm the method and result shown in this protocol, future work should include the selection of more stressors and evaluation methods for CUMS method and the detection of the longer-term effects of Xiaoyaosan for 28–48 days of CUMS.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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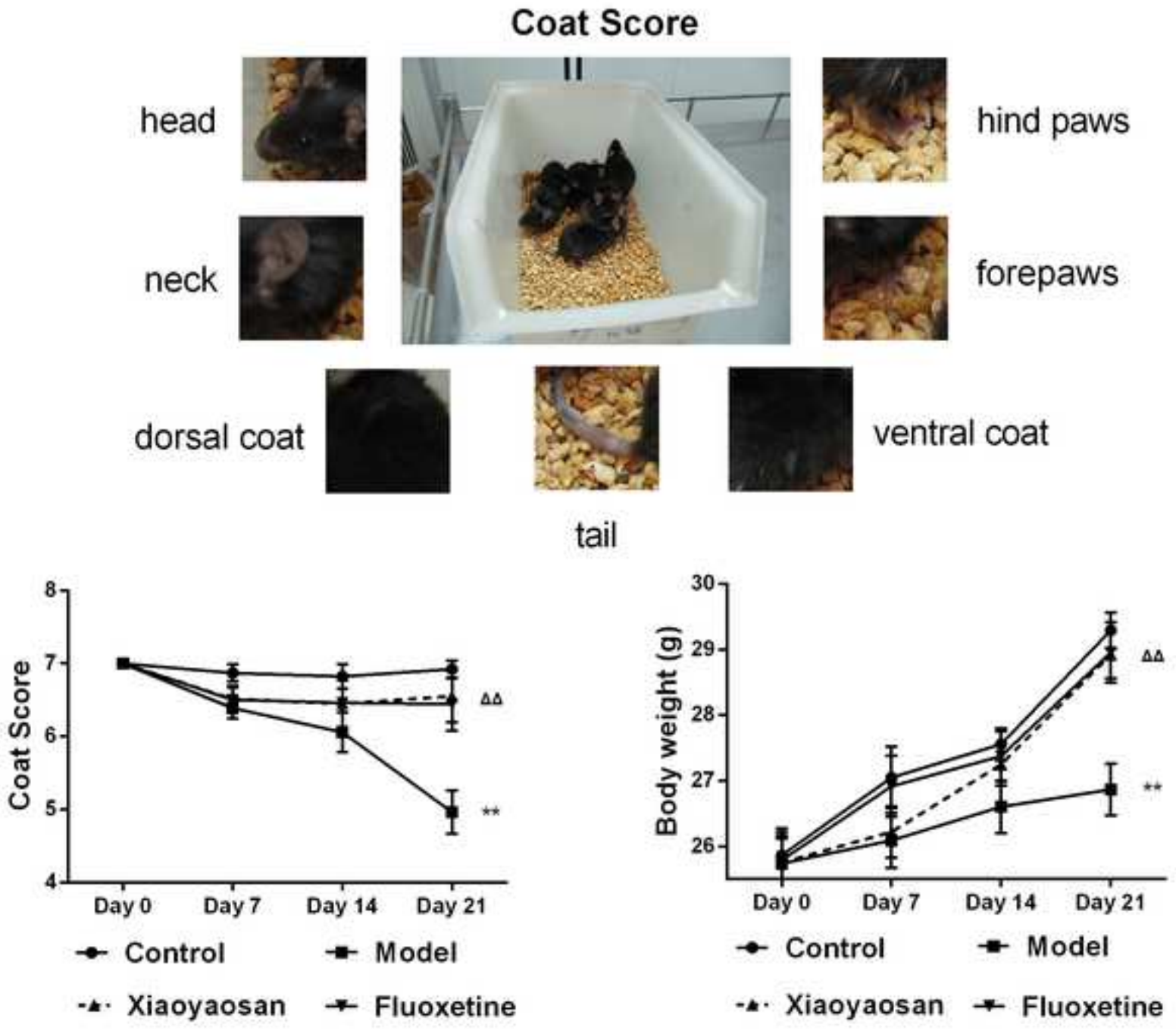

Figure

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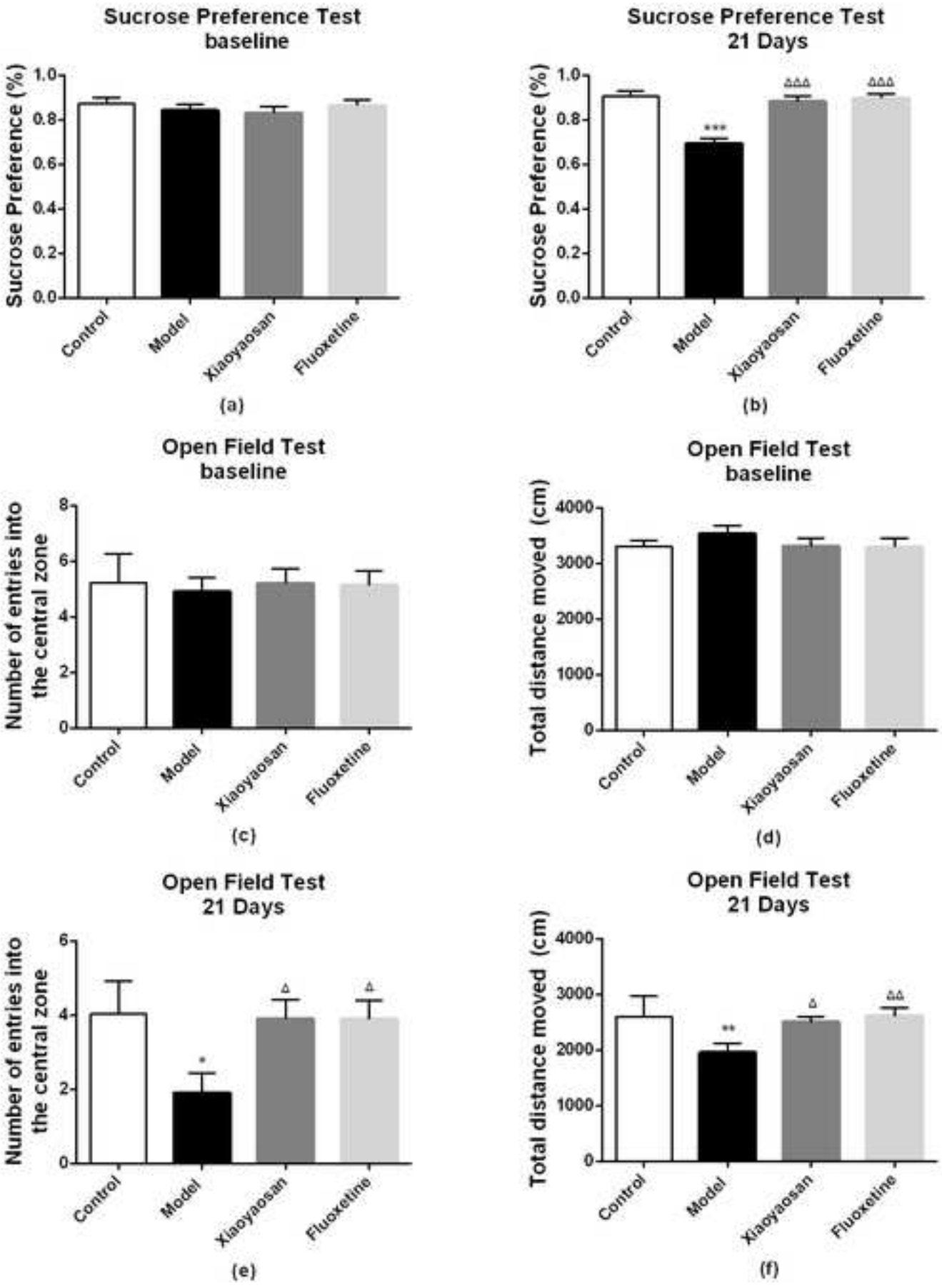

Table of Materials

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JoVE_Materials_062818.xlsx

Name of Material/ Equipment
Chinese Herbs
Fluoxetine
C57BL/6j mice
Mouse cage
Distilled Water
Sucrose
ACS-2EAS Electronic Balance
Wooden Black Lusterless Box
WV-CP470 Camera
Etho Vision 3.0 Software
SPSS Statistics 21.0 Software
Graphpad Prism 6.0 Software

Company	Catalog Number
Tongrentang (Bozhou, AnHui) Decoction Pieces Limited Company	-
PATHEON FRANCE	5545A
Beijing Vital River of the Charles River Company Styling Biotechnology	SCXK 2011-0004 34853016
-	-
Sigma	-
Beijing Hengzhongheng Electronic Technology Co., Ltd.	-
-	-
Panasonic, Japan	-
Noldus, Wageningen, Holland	-
IBM	-
Graphpad Software,Inc.USA	-

Comments/Description

preparation room of the China-Japan Friendship Hospital, distilled water was used to prepare the suspe

The distilled water was used to prepare the suspension of fluoxetine, and the dose 2.6 mg/kg/d.

thy male C57BL/6J mice aged 12 weeks were purchased from Beijing Vital River of the Charles River Cor
318×202×135 mm

The water was used througout the experiment.

The sucrose was used in the sucrose preference test to prepare 1% sucrose solution.

It was used to weigh the avoirdupois of mice at different stages.

(40 cm x 40 cm x 15 cm), divided into 25 equal squares by blue lines.

A HD digital camera which was used for recording the whole process of the open field test.

A video-tracking system which was used to evaluate the locomotor function and emotionality of mice.

on-parametric test was used for general data based on normality test and homogeneity test for varianc

Graphpad Prism 6.0 software was used to draw statistical graphs.

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Title of Article: Compound Prescription Xiaoyaosan Improves Depressive-Like Behaviors of Chronically Stressed Mice

Author(s): Zhi-Yi Yan, Xiao-Juan Li, Xiu-fang Ding, Yue-yun Liu, and Jia-xu Chen

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Article Title: Compound Prescription Xiaoyaosan Improves Depressive-Like Behaviors of Chronically Stressed Mice

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Response to Editorial comments

Thank you for your comments concerning our manuscript entitled 'Compound Prescription Xiaoyaosan Improves Depressive-Like Behaviors of Chronically Stressed Mice' (JoVE58276). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. The comments were all valuable and very helpful for revising and improving our paper and the important guiding significance of our research. We have carefully considered the comments and addressed all of your concerns, which we hope meet with approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the comments are as flowing:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: We have studied comments carefully and have made correction. We hope it will meet with approval, and we will search for language polishing by a native speaker if the contents of our manuscript are suitable for publication.

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Response: The missing citation has been added in the Figure Legend.

3. Figures: Please define SEM in the figure legend.
Response: We are very sorry for the mistake. The missing definition of SEM has been added into the revised manuscript.

4. Please provide an email address for each author.
Response: The missing email address for each author has been added into the revised manuscript.

5. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
Response: The Short Abstract has been improved in the revised manuscript. We hope it would meet with approval.

6. Please revise the Long Abstract to focus on the method being presented rather than the results of a specific experiment. Include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.
Response: The Long Abstract has been improved in the revised manuscript. We hope it would meet with approval.

7. Some information in the Introduction doesn't appear to be broadly-supported (e.g., that xiaoyaosan "dispers[es] … stagnant liver-energy and nourish[es] blood"); please revise.

Response: Thank you for pointing out this problem. The citation has been added into the revised manuscript.

8. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Tongrentang, Beijing Vital River of the Charles River Company, EthoVision, etc.
Response: It has been revised in the manuscript, thank you very much.

9. Please define all abbreviations before use.
Response: The abbreviations have been defined in the revised manuscript.

10. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
Response: The manuscript has been improved, we hope it meets with approval, thank you very much.

11. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section (e.g. lines 171-178).
Response: It has been revised in the manuscript.

12. 2.1: Please provide the dimension for the cage.
Response: It has been added in the JoVE Materials file, thank you very much.

13. 4.1: Please describe how to prepare the suspension of Xiaoyaosan and fluoxetine.
Response: It has been improved in the manuscript.

14. 6.1: How is the video-tracking system set up, i.e., where is it positioned, on the top or from the side?
Response: It has been improved in the manuscript.

15. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.
Response: It has been revised in the manuscript, thank you very much.

16. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a. Critical steps within the protocol
b. Any modifications and troubleshooting of the technique
c. Any limitations of the technique
d. The significance with respect to existing methods
e. Any future applications of the technique

Response: It has been improved in the manuscript.

17. References: Please do not abbreviate journal titles.
Response: It has been revised in the manuscript, thank you very much.

Response to Reviewer 1 Comments

Thank you for your comments concerning our manuscript entitled 'Compound Prescription Xiaoyaosan Improves Depressive-Like Behaviors of Chronically Stressed Mice' (JoVE58276). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches.

In consideration of your three main concerns, we have the following explanation.

First, the compounds of Xiaoyaosan used in the protocol has a good effect on depression which has been proved in our previous experimental studies (Yue G X; Huang Q F; Chen J.X. Antidepressant effects of Xiaoyao powder and its different modified combinations. World J. Integr. Tradit. Western Med. 2007, 6, 326–328; Ding X F, Li Y H, Chen J X, et al. Involvement of the glutamate/glutamine cycle and glutamate transporter GLT-1 in antidepressant-like effects of Xiao Yao san on chronically stressed mice [J]. Bmc Complementary & Alternative Medicine, 2017, 17(1):326), so it could be used in this protocol for observing the antidepressant effect of Traditional Chinese Medicine.

Second, thank you very much for your concern about the use of previous data that have been published in this manuscript. The purpose of this protocol is to exhibit the methods in the article *Involvement of Normalized Glial Fibrillary Acidic Protein Expression in the Hippocampi in Antidepressant-Like Effects of Xiaoyaosan on Chronically Stressed Mice* by Dr. Xiu-Fang Ding, et al, so we could present the corresponding data in the manuscript if cited. We have obtained explicit copyright permission to reuse data from this previous publication.

In the end, we are sorry for the language mistakes in our manuscript. Now we have studied comments carefully and have made correction which we hope meet with approval, and also have checked grammar, spelling, and punctuation in the manuscript, and we will search for language polishing by a native speaker if the contents of our manuscript are suitable for publication.

The main corrections in the paper and the responds to the comments are as flowing:

Point 1: What do you mean by using the item"Compound prescription Xiaoyaosan"? do you mean the compounds that are contained in Xiaoyaosan? Please explain.

Response 1: The 'Compound prescription Xiaoyaosan' in the manuscript is the meaning of ' the compounds that are contained in Xiaoyaosan'. Thank you for pointing out this incomprehensible phrase, the 'Compound prescription Xiaoyaosan' has been revised in the manuscript.

Point 2: The title does not match the research content very well. I would like to suggest to change it as "Compounds of Xiaoyaosan prescription improves depressive -like behaviors of chronically stressed mice".

Response 2: Thanks for your valuable advice, the title has been replaced to 'Compounds of Xiaoyaosan prescription improves depressive -like behaviors of chronically stressed mice' in the revised manuscript.

Point 3: In the abstract, "a series of data"should be changed as "a series of parameters".

Response 3: Thanks for your advice, the 'a series of data' in the long abstract has been replaced to 'a series of parameters' in the revised manuscript.

Point 4: The description in the manuscript is poor. I changed the last part of the abstract as an example.
The original description: "Through data analysis after the experiment, significant behavioral changes were found in model mice, meanwhile the depressive like behaviors could be improved by Xiaoyaosan or fluoxetine treatment. This protocol demonstrated the application of Xiaoyaosan to the behaviors of CUMS-induced mice, and revealed that the beneficial effects of Chinese compound prescription Xiaoyaosan on depressive-like behaviors may be worth considering for the treatment of depression."
My modification："Data analysis showed that behaviors of model mice were significantly changed as compared to control group, which however have been improved by the treatment of Xiaoyaosan and fluoxetine. The current findings demonstrated the anti-depression effects of Xiaoyaosan on the behaviors of CUMS-induced mice, and revealed that compounds from Xiaoyaosan prescription may be worth for treating depression taking into account their beneficial effects on depressive-like behaviors."

Response 4: Thank you for your thoughtful comments. We are so sorry for the language deficiencies and problems in our manuscript. We have made correction which we hope meet with approval, and we will search for language polishing by a native speaker if the contents of our manuscript are suitable for publication in the in the next round of modification.

Point 5: Line 88, give the explanation of abbreviation of "XYS" somewhere previously.

Response 5: Thank you for your careful work. This is a wrong abbreviation and it is revised in the new manuscript.

Point 6: All numbers that are smaller than ten should be spelt out, for instance, one week, five-sucrose preference test, any two of the following seven stressors, etc.

Response 6: Thank you very much for this constructive comment. According to your suggestion, all numbers that are smaller than ten have been spelt out in the revised manuscript.

Point 7: Line 137-138, did you perform the open filed test and sucrose preference test prior to the experiment? If yes, please give the data and compare the results obtained before and after the experiments. By doing so, you would be able to confirm the model has been built successfully.

Response 7: In this protocol, the open filed test and sucrose preference test were performed before and after the process of model establishment, the description of the open filed test and sucrose preference test prior to the experiment was in the section of '2. Preparing Mice for the Experimental Procedure', and the corresponding data were presented in the section of 'REPRESENTATIVE RESULTS'. Thank you for your thoughtful comments!

Point 8: In all parts of results, the authors have not compared the effects of XYS to the effects of fluoxetine. It is better to do it to give a direct idea how strong the anti-depression effect of XYS is.

Response 8: Thank you for your thoughtful advice. To further verify the correctness of the depression model, we set the fluoxetine treatment group in this study. The purpose of our experiment is to find the antidepressant effect of Xiaoyaosan, rather than the advantage of traditional Chinese medicine in antidepressant. Furthermore, the efficacy comparison between

Xiaoyaosan and fluoxetine is not common in previous studies (Zhu X, Jing L, Chen C, et al. Danzhi Xiaoyao San ameliorates depressive-like behavior by shifting toward serotonin via the downregulation of hippocampal indoleamine 2, 3-dioxygenase[J]. Journal of Ethnopharmacology, 2015, 160:86-93; Zhu X, Xia O, Han W, et al. Xiao Yao San Improves Depressive-Like Behavior in Rats through Modulation of β-Arrestin 2-Mediated Pathways in Hippocampus[J]. Evid Based Complement Alternat Med, 2014, 2014(6):902516). We would have an in-depth discussion in the manuscript if the anti-depression effect of Xiaoyaosan had significant advantage to fluoxetine according to statistical analysis.

Point 9: The formats of references are not consistent. For instance, in references 12 and 13, the words were capitalized while for other references the words in the titles were not. Please follow the instruction rules of the journal.

Response 9: Thank you for your careful reading of our manuscript. We have careful rechecked and corrected the style of the citations according to the requirement of JoVE.

Point 10: The figures are not in a good quality and should be improved.

Response 10: Thank you for your careful work. We are very sorry for the low quality figures in the paper. We have corrected it in the revised manuscript.

Minor Concerns The language needs to be improved. Here I corrected some of them.
1. In the abstract, "Mice were randomly divided into 4 groups, they were control group, model group, Xiaoyaosan treatment group, and fluoxetine treatment group." should be changed as "Mice were randomly divided into 4 groups, i.e. control group, model group, treatment group of Xiaoyaosan, and treatment group of fluoxetine."
2. In the abstract, "after the experiment" should be deleted.
3. Line 17, change "the intervention effect" into "the anti-depression effect"
4. Line 41," It has become the world's primary cause of disability," should be changed as "It has become one of the world's primary causes of disability,"
5. Line 44-46, it is better to divide this sentence into two independent sentences.
6. Line 47, delete "compound".
7. Line 53, add references related for the statement "Containing multiple constituents, Xiaoyaosan has multiple targets, and utilize multiple pathways."
8. Line 69, "the mouse depressive model" should be changed as "the depressive model of mouse"
9. Line 68-73, it is too tedious to express your research aim in one sentence. Please re-written this part.
10. Line 115-127, add "a.m." after the time points.
11. Line 145-146, it is better to give the ratios of the eight herbs contained in Xiaoyaosan. Did you use the original ratios or did you modify the ratios? It is not clear.
12. Line 175, what does "HD" means?
13. Line 186, which statistical software?
14. Line 223, you mentioned there will be "FIGURE & TABLE LEGENDS". However, no table legends were provided. Please check.
15. The references in the current study are not enough. For instance, give some references related to the statement between Line 235-240, between 244-246 in the discussion part.
16. Line 254, "state" should be "states"
17. Line 270-271, this sentence should be a new sentence.
18. Line 271, "Many study has…" should be "many studies have…"

Response: Thank you very much to point out these issues in our manuscript. We have careful rechecked and corrected the above problems according to your profound comments. We hope our revised manuscript could meet publication requirements, and we will seek for the professional polishing. Thank you very much!

Response to Reviewer 2 Comments

Thank you for your comments concerning our manuscript entitled 'Compound Prescription Xiaoyaosan Improves Depressive-Like Behaviors of Chronically Stressed Mice' (JoVE58276). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. The comments were all valuable and very helpful for revising and improving our paper and the important guiding significance of our research. We have carefully considered the comments and addressed all of your concerns. We are sorry for the language mistakes in our manuscript. Now we have studied comments carefully and have made correction which we hope meet with approval, and we will search for language polishing by a native speaker if the contents of our manuscript are suitable for publication. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the comments are as flowing:

Point 1: The depression animal model established by chronic unpredictable mild stress has been widely accepted in the field, and numerous studies have been published regarding the effect of Xiaoyaosan on depression both in human and animal model, including the works of the authors themselves. What is new finding of the present manuscript?

Response 1: Thank you for your thoughtful concern about our manuscript. The purpose of this protocol is to exhibit the methods in the article *Involvement of Normalized Glial Fibrillary Acidic Protein Expression in the Hippocampi in Antidepressant-Like Effects of Xiaoyaosan on Chronically Stressed Mice* by Dr. Xiu-Fang Ding, et al. We hope our manuscript could explain the methodological part of the article mentioned here in a more comprehensive way, and meet with approval from JoVE, then this protocol would be recorded in the form of video.

Point 2: The data presented in this manuscript are concerning the preventive effect of Xiaoyaosan on the development of depression, which has little, if any, clinical relevance, since it is hard to image a health people taking a medicine for an unpredictable long time to prevent a potential depression.

Response 2: Thank you for your valuable advice. A health people taking a medicine for an unpredictable long time to prevent a potential depression is not suitable in clinical practice. But the approach of prophylactic treatment by medicine to study the mechanism of action is widely used in many animal experiments (Liu X J, Zhou Y Z, Li Z F, et al. *Anti-depressant effects of Xiaoyaosan on rat model of chronic unpredictable mild stress: a plasma metabonomics study based on NMR spectroscopy* [J]. Journal of Pharmacy & Pharmacology, 2012, 64(4):578-588; Ramakers J D, Verstege M I, Thuijls G, et al. *The PPARγ Agonist Rosiglitazone Impairs Colonic Inflammation in Mice with Experimental Colitis* [J]. Journal of Clinical Immunology, 2007, 27(3):275-283; Yue N, Huang H, Zhu X, et al. *Activation of P2X7 receptor and NLRP3 inflammasome assembly in hippocampal glial cells mediates chronic stress-induced depressive-like behaviors* [J]. Journal of Neuroinflammation, 2017, 14(1):102).
Xiaoyaosan, as an herbal prescription, has been used to treat mental disorders for a long time. So, this protocol described the establishment of mouse depressive model and evaluated the preventive and treatment effect of Xiaoyaosan to the depressive-like behaviors of chronically stressed mice.

Point 3: More importantly, the authors stated that "The protocol and results demonstrated in this paper originates from the article "Involvement of Normalized Glial Fibrillary Acidic

Protein Expression in the Hippocampi in Antidepressant-Like Effects of Xiaoyaosan on Chronically Stressed Mice by Dr. Xiu-Fang Ding, et al." (line 188-190, and line 277-280). The paper the authors referred to has been published, as ref.13 in the list of references of this manuscript. Therefore, the data of the present manuscript, nevertheless, should not be considered for publication again.

Response 3: Thank you very much for your concern about the use of previous data that have been published in our manuscript. The purpose of this protocol is to exhibit the methods in the article *Involvement of Normalized Glial Fibrillary Acidic Protein Expression in the Hippocampi in Antidepressant-Like Effects of Xiaoyaosan on Chronically Stressed Mice* by Dr. Xiu-Fang Ding, et al, we have obtained explicit copyright permission to reuse data from this previous publication. We could present the corresponding results in the manuscript if cited.

Numerous irregular expressions and formats exist throughout the manuscript, in addition to the errors in grammar, which the reviewer will not detail given the concerns above.

Response: Thank you very much to point out these issues in our manuscript. We have careful rechecked and corrected the language problems in our manuscript. We hope our revised manuscript could meet publication requirements, and we will seek for the professional polishing. Thank you so much!

Response to Editorial comments

Thank you for your comments concerning our manuscript entitled 'Evaluating the anti-depression effect of the Xiaoyaosan prescription on chronically-stressed mice' (JoVE58276). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. The comments were all valuable and very helpful for revising and improving our paper and the important guiding significance of our research. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the comments are as flowing:

Changes to be made by the Author(s):

1. There are still numerous errors in grammar and usage; please revise, ideally by a fluent English speaker.

Response: Thanks for your valuable advice, we have revised the grammar and usage by a native speaker, and we hope our manuscript would be suitable for publication. Thanks again!

2. Title: This should reflect the protocol more as opposed to the results; e.g., "Evaluating the anti-depression effect of Xiaoyaosan on chronically-stressed mice".

Response: Thanks for your comment, the title has been replaced to 'Evaluating the anti-depression effect of the Xiaoyaosan prescription on chronically-stressed mice' in the revised manuscript.

3. Introduction: Please tone down overly broad statements on the safety, efficacy, and physiological effects of Xiaoyaosan, and/or provide more citations.

Response: Thank you very much for this constructive comment. According to your suggestion, we have added citations [8, 9, and 10] in the Introduction section of the manuscript as the support for the effects of Xiaoyaosan.

4. Please rewrite much of the protocol in the imperative tense (e.g., "Keep mice individually in cages". "Use distilled water", etc.). Any text that cannot be written in the imperative should be a 'note'.

Response: It has been revised in the manuscript, thank you very much.

5. 3.1: Please provide more detail or citations about how to carry out these stressors. Also, could you explicitly say what the seven stressors.

Response: The protocol has been improved in the manuscript, we hope it would meet with approval. Thank you!

6. 5.3: 'The last day' and '48 h' seem contradictory.

Response: Thank you for your careful work. This is a mistake and it is revised in the new manuscript.

7. Figure 2: The panels seem to be mislabeled in the legend.

Response: Thank you for pointing out this problem. It has been revised in the manuscript.

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 This information has been uploaded to the Editorial Manager. Thank you for your thoughtful advice.

9. Discussion: This should be rewritten from the perspective of researchers wanting to replicate this protocol, including critical steps, troubleshooting measures, possible modifications, limitations, comparisons to other similar protocols, and future applications.

Response: Thank you very much for this constructive comment. According to your suggestion, this section has been improved in the revised manuscript.

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