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Synergistic Antidepressant Effects of Total Saikosaponins Combined with Volatile Oil of Cyperi Rhizoma in Mice Models Induced by Chronic Restraint plus Mild Stress

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Abstract: This study aimed to evaluate the antidepressant effects of the combined administration of total saikosaponins (SSA) and volatile oil of Cyperi Rhizoma (VO) using a mouse depression model induced by chronic restraint plus mild stress (CRMS), and to compare the effects with the traditional antidepressant fluoxetine. Male Kunming mice were subjected to 14-day CRMS modeling and then randomly divided into four groups: the combined treatment group (intraperitoneal injection of SSA 3.5 $mg \cdot kg^{-1}$ + VO 35 $mg \cdot kg^{-1}$), the fluoxetine treatment group (20 $mg \cdot kg^{-1}$), the normal saline treatment group, and the non-model group. Drugs were administered continuously for 14 days. Depressive-like behaviors were assessed using the Forced Swimming Test (FST), Tail Suspension Test (TST), and Open-Field Test (OFT). The results showed that the absolute immobility time of mice in the CRMS model group was significantly prolonged in FST and TST. Combined administration of SSA and VO significantly improved depressive-like behaviors, restoring the absolute immobility time in FST and TST to levels close to the control group, with efficacy comparable to fluoxetine. This study confirms that the combination of SSA and VO exhibits antidepressant effects equivalent to fluoxetine in the CRMS model, providing experimental evidence for the further clinical development of this traditional Chinese medicine (TCM) compatibility.

Keywords: Total saikosaponins; Volatile oil of *Cyperi Rhizoma*; Combined administration; Depression; Chronic restraint plus mild stress

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1. Introduction

Major depressive disorder (MDD) is a mental illness characterized by persistent low mood, anhedonia, cognitive impairment, and various somatic symptoms. The global lifetime prevalence of MDD is as high as

19.6%, with more than 300 million patients worldwide and approximately 58 million in China (WHO, 2022) [1]. It is predicted that by 2030, MDD will become the leading cause of premature death and disability [2]. Although selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are widely used [3, 4], 30%–50% of patients still suffer from treatment-resistant depression. Additionally, these drugs have drawbacks such as delayed onset (2–4 weeks), sexual dysfunction, gastrointestinal disorders, insomnia, and arrhythmia in the elderly [3]. Therefore, there is an urgent need to explore new therapeutic strategies with rapid onset, minimal side effects, and suitability for long-term use.

TCM, characterized by "multi-component, multi-target, and multi-pathway" effects, provides new ideas for the treatment of depression. In TCM, depression is categorized as "stagnation syndrome", with the core pathogenesis of "liver qi stagnation" and the therapeutic principle of "soothing the liver and relieving stagnation". The drug pair of Bupleuri Radix et Rhizoma and Cyperi Rhizoma is present in more than 60% of antidepressant compound prescriptions, such as Chaihu Shugan San recorded in Jingyue Quanshu (Complete Works of Jingyue). Bupleuri Radix et Rhizoma is slightly cold in nature and belongs to the liver meridian. Its main active components, saikosaponin A and D, exert antidepressant effects by upregulating the hippocampal BDNF-TrkB-CREB pathway [5], inhibiting NF-κB-mediated neuroinflammation [6], and regulating the hypothalamic-pituitary-adrenal (HPA) axis function [7, 8]. Cyperi Rhizoma is neutral in nature, regulating qi and soothing the liver. Its volatile oil is rich in α-cyperone, cyperone, and sesquiterpene oxides, with multiple effects such as inhibiting acetylcholinesterase, enhancing 5-HTergic neurotransmission, and improving anxiety-like behaviors induced by chronic stress [8, 9]. Previous network pharmacology predictions by the research team indicated that saikosaponins and volatile oil of Cyperi Rhizoma act on 10 core targets including 5-HT1A, BDNF, mTOR, and AChE, suggesting potential synergistic effects. However, systematic reports on the compatibility ratio, dosage window, preventive/therapeutic properties, and mechanism verification of the key active components of this drug pair are lacking. Bupleuri Radix et Rhizoma-Cyperis Rhizoma is a classic TCM pair for "soothing the liver and relieving stagnation", widely used clinically in emotional disorders. Nevertheless, standardized experimental evidence for the synergistic antidepressant mechanism between its main active components—total saikosaponins (SSA) and volatile oil of Cyperi Rhizoma (VO)—is insufficient.

This study adopted the chronic restraint plus mild stress (CRMS) model, which has good face validity, construct validity, and predictive validity [10], and can simulate depression caused by psychosocial stress in humans [11]. Through behavioral, body weight, acute toxicity, and histological evaluations, this study systematically explored the optimal dosage, therapeutic and preventive effects of SSA + VO, and conducted a head-to-head comparison with the positive drug fluoxetine, providing experimental evidence for the modernization of this classic drug pair.

2. Materials and methods

2.1. Medicinal materials, reagents, and instruments

Cyperi Rhizoma decoction pieces were purchased from Bozhou, Henan (Batch No. 20230401). Volatile oil was extracted by steam distillation for 2.5 h according to Method A in the "Determination of Volatile Oil" appendix of the 2020 Edition of the Chinese Pharmacopoeia (Part IV), yielding a pale yellow volatile oil (yield 0.41%, d_{20}^{20} 0.981). GC-MS identification showed the main components as α-cyperone (18.7%), cyperone (14.2%), and cyperene (11.5%) (Supplementary Table S2). Total saikosaponins (≥70%, containing 38% SS-A and 22% SS-

D) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Batch No. Z30O11L129400). Fluoxetine hydrochloride was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Cat. No. F830634).

2.2. Experimental animals and feeding conditions

SPF-grade male Kunming mice, 8 weeks old, weighing 20–25 g, were provided by Guangdong Provincial Medical Experimental Animal Center [SCXK (Guangdong) 2022-0002]. After 7 days of adaptive feeding, mice were raised at room temperature (22±2°C), humidity (45–55%), and a 12 h light-dark cycle, with free access to food and water. The experiment was approved by the Animal Ethics Committee of Jiaying University (JYAE2023-03).

2.3. Extraction and content determination of volatile oil

Referring to the pharmacopoeia method: 100 g of crushed Cyperi Rhizoma was soaked in 3 times the volume of water for 18 h, placed in a 1000 ml round-bottomed flask, connected to a volatile oil determination apparatus, and distilled at a gentle boil until no further increase in oil volume (approximately 5 h). The volatile oil was collected, centrifuged at 13000 r/min for 5 min, and the upper oil phase was stored at 4°C in the dark.

2.4. CRMS modeling protocol

On the first day, mice were restrained for 2 h, with the restraint time increased by 2 h daily until reaching 8 h, which was then maintained. Meanwhile, one mild stressor was randomly applied daily: ① 16 h fasting and water deprivation; ② wet bedding; ③ cage tilted at 45°; ④ day-night reversal. Modeling was conducted continuously for 14 days, with a success rate >90% (≥40% increase in FST immobility time).

2.5. Grouping and administration

Phase I: Dosage Exploration (n=24)

Three dosage combinations were set according to 1/10, 1/20, and 1/40 of LD_{50}: SSA 7, 3.5, 2.3 $mg \cdot kg^{-1}$ + VO 70, 35, 17.5 $mg \cdot kg^{-1}$. Deaths and skin stiffness occurred in the high-dose group (7+70) from day 7, while the medium-dose group (3.5+35) showed no abnormalities within 14 days and optimal behavioral performance, which was determined as the dosage for subsequent experiments.

Phase II: Therapeutic Experiment (Post-CRMS, n=36)

After modeling, mice were randomly divided into four groups: ① Control + normal saline (Group C); ② Model + normal saline (containing 6.95% ethanol vehicle) (Group M); ③ Model + fluoxetine 20 $mg \cdot kg^{-1}$ (Group MF); ④ Model + SSA + VO (3.5+35 $mg \cdot kg^{-1}$) combined administration (Group CD). Drugs were administered once daily for 14 consecutive days.

2.6. Behavioral tests

(1) Forced Swimming Test (FST)

The test was conducted in buckets (height 30 cm, width 12 cm) filled with 22 cm deep water. Four mice could be tested simultaneously, separated by opaque partitions. In this test, the time when mice ceased struggling, floated with only slight limb movements to keep their heads above water was defined as absolute immobility time. Longer immobility time indicated more severe depression.

(2) Tail Suspension Test (TST)

Mice were gently removed from cages, and their tails were fixed with medical adhesive tape 1 cm from the tip to avoid additional stress. The monitoring index was absolute immobility time: the time when animals ceased struggling and remained vertically suspended motionless. Longer immobility time indicated more severe depression.

(3) Open-Field Test (OFT)

The test was performed in square boxes (bottom diameter 47.5 cm, wall height 47.6 cm), with four boxes used simultaneously. Total activity distance reflected the spontaneous activity level and vitality of mice, while central activity distance indicated stronger desire to explore new environments and lower anxiety levels.

All experiments were recorded and analyzed using Smart 3.0 professional behavioral software. The monitoring indices for FST and TST were absolute immobility time within 5 min, and those for OFT were total activity distance and central activity distance.

2.7. Statistical analysis

SPSS 25.0 software was used for statistical analysis of experimental data. Multiple group comparisons were performed using analysis of variance (ANOVA) followed by LSD post-hoc test. A p-value <0.05 was considered statistically significant.

3. Results

3.1. FST and TST results

As shown in **Figure 1**A (FST results), compared with Group C (71.67±9.43), the absolute immobility time of Group M (108.60±14.18) was significantly increased, with a statistically significant difference. Compared with Group M, the absolute immobility time of Group CD (71.66±8.73) and Group MF (72.75±9.21) was significantly reduced, with statistically significant differences, and the immobility time of Group CD was comparable to that of Group MF. No significant difference in absolute immobility time was observed between Group CD, Group MF, and Group C. In summary, combined administration of SSA + VO reduced the absolute immobility time of CRMS model mice, with effects equivalent to fluoxetine.

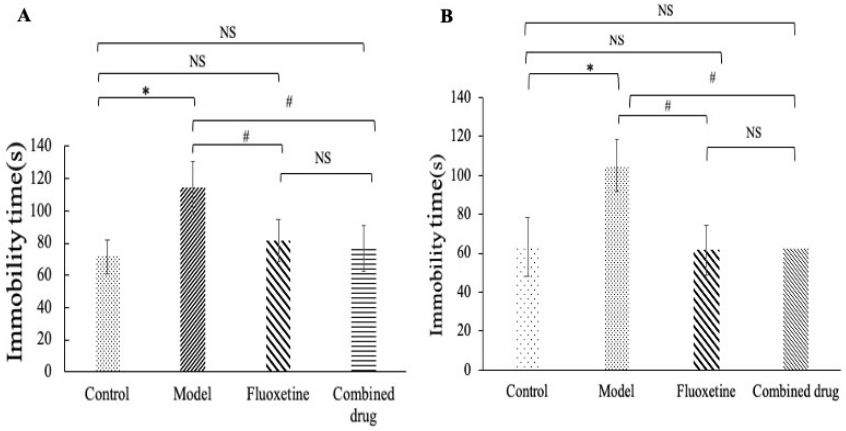

Figure 1. FST and TST tests after combined drug treatment

Note: ** indicates $p < 0.05$ compared with Group C; # indicates $p < 0.05$ compared with Group M; NS indicates no significance.*

As shown in **Figure 1**B (TST results), compared with Group C (63.19±10.53), the absolute immobility time of Group M (104.97±15.12) was significantly increased, with a statistically significant difference. Compared with Group M, the absolute immobility time of Group CD (62.57±12.69) and Group MF (61.59±13.46) was significantly reduced, with statistically significant differences, and the immobility time of Group CD was comparable to that of Group MF. No significant difference in absolute immobility time was observed between Group CD, Group MF, and Group C. In summary, combined administration of SSA + VO reduced the absolute immobility time of CRMS model mice, with effects equivalent to fluoxetine.

3.2. Open-field test (OFT) results

As shown in **Figure 2** (OFT results), there was no significant difference in total activity distance among the four groups. However, regarding central activity distance, compared with Group C (363.19±12.53), the central activity distance of Group M (224.87±17.32) was significantly reduced, with a statistically significant difference. Compared with Group M, the central activity distance of Group CD (330.59±15.66) and Group MF (370.22±14.69) was significantly increased, with statistically significant differences, and the central activity distance of Group CD was comparable to that of Group MF. No significant difference in central activity distance was observed between Group CD, Group MF, and Group C. In summary, combined administration of SSA + VO significantly increased the central activity distance of the model group, with effects equivalent to fluoxetine.

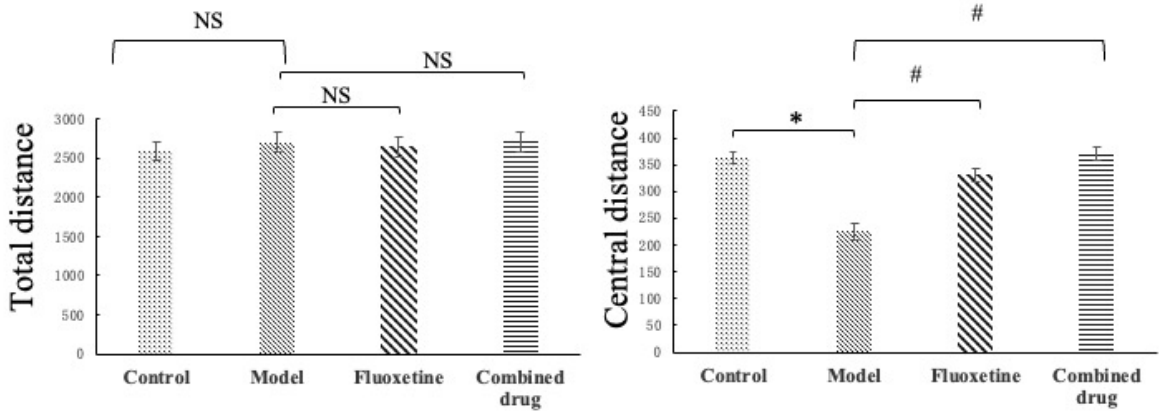

Figure 2. OPT test after combined medication treatment

Note: ** indicates $p < 0.05$ compared with Group C; # indicates $p < 0.05$ compared with Group M; NS indicates no significance.*

4. Discussion

This study is the first to systematically evaluate the antidepressant effects of the combination of SSA and VO in a mouse model of chronic restraint plus mild stress (CRMS). Behavioral tests (FST, TST, and OFT) showed that combined administration of SSA and VO restored the absolute immobility time or central activity distance of CRMS model mice to normal levels, with efficacy equivalent to the clinical first-line antidepressant fluoxetine (20 $mg \cdot kg^{-1}$). This finding provides modern experimental evidence for the classic liver-soothing and stagnation-relieving drug pair "Bupleuri Radix et Rhizoma-Cyperis Rhizoma" and suggests the potential of multi-

component botanical drugs to replace chemical drugs in the intervention of depression.

The synergistic effects of SSA and VO may affect the "monoamine-neurotrophic-cholinergic" network. First, saikosaponin A and D in SSA have been confirmed to activate the hippocampal mTORC1-PSD95 signaling cascade within 6 hours, rapidly increasing postsynaptic density protein levels, thereby reversing synaptic atrophy induced by chronic stress [7]. In this study, the SSA + VO group showed antidepressant effects equivalent to fluoxetine after 14 days of single daily administration, consistent with the above "rapid onset" finding. Second, α-cyperone, abundant in VO, can enhance GABAA receptor function, reduce excessive excitation of the basolateral amygdala, and alleviate anxiety-like behaviors [12]; meanwhile, VO can inhibit acetylcholinesterase activity, reduce acetylcholine decomposition, thereby improving stress-related cognitive rigidity [13, 14]. In the OFT, we observed that SSA + VO significantly increased central activity distance, suggesting dual anti-anxiety and antidepressant properties, consistent with the cholinergic regulatory effect of VO. Third, the joint upregulation of the BDNF-TrkB pathway by both components is supported by proteomic evidence: Gurtoo et al. found that BDNF was the most significantly elevated differential protein in the serum of hypoxic-ischemic neonates [5], and SS-A enhances neuroplasticity precisely through the TrkB receptor [5,15]. Therefore, SSA and VO affect the "monoamine-neurotrophic-cholinergic" three-dimensional network at the molecular level, synchronously alleviating emotional, cognitive, and somatic symptoms, and making up for the deficiency of fluoxetine's single target.

However, this study has the following limitations: first, only male mice were used, lacking data on gender differences; second, although the behavioral experimental results are sufficient and repeatedly verified, serum or hippocampal levels of 5-HT, DA, BDNF, and AChE were not detected, and the mechanism still needs verification by ELISA or Western blotting. Finally, although CRMS can simulate psychosocial stress, it lacks direct indicators of anhedonia, a core symptom of human depression. In subsequent studies, the sucrose preference test can be combined to further confirm the improvement of reward dysfunction.

In conclusion, the combination of SSA and VO exhibits antidepressant effects equivalent to fluoxetine with higher safety in the CRMS model. Its "multi-component, multi-target, multi-pathway" characteristics provide new ideas for overcoming the shortcomings of traditional monoamine drugs. Future research should further explore the molecular mechanisms and optimize oral formulations, and promote early intervention clinical trials in high-risk populations to realize the modernization and international promotion of this classic drug pair.

5. Conclusions

The chronic restraint plus mild stress model can stably induce depressive-like behaviors in mice, suitable for evaluating antidepressant efficacy.

The combination of SSA (3.5 $mg \cdot kg^{-1}$) and VO (35 $mg \cdot kg^{-1}$) can improve depressive-like behaviors in CRMS model mice, with effects equivalent to fluoxetine.

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Disclosure statement

The authors declare no conflict of interest.

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