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Original Research Article

Oridonin ameliorates depressive-like behaviors induced by chronic unpredictable mild stress in mice via TXNIP/NLRP3 signaling pathway

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Abstract

Purpose: *To investigate the effect and mechanism of oridonin in chronic unpredictable mild stress (CUMS)-induced depressive-like behaviors.*
Methods: *CUMS was established using 6-week stress stimuli, including feed/water deprivation, night lighting, inverted light/dark cycle, and tail clamping. Depressive behaviors were analyzed using the sucrose preference test, forced swim test (FST), and tail suspension test (TST). Locomotor activity was analyzed using the open field test (OFT) while inflammatory cytokines were analyzed by enzyme-linked immunosorbent assay. The activation of the TXNIP/NLRP3 signaling pathway was evaluated by western blot.*
Results: *Sucrose consumption of CUMS-treated mice was significantly decreased, while immobility times of the FST (control vs. CUMS, ~50 to 150 s; $p < 0.01$) and TST (Control vs. CUMS, ~50 to 130 s; $p < 0.01$) were increased; oridonin significantly reversed these effects. Spontaneous locomotor activities (crossing, rearing, and grooming) measured in the OFT were decreased after the CUMS procedure, and oridonin increased these activities ($p < 0.01$ vs. CUMS). Oridonin decreased the production of tumor necrosis factor alpha, interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein-1 in the hippocampus of CUMS-treated mice and significantly inhibited activation of the TXNIP/NLRP3 pathway induced by CUMS.*
Conclusion: *Oridonin ameliorates depressive-like behaviors in mice induced by CUMS, partly via TXNIP/NLRP3 signaling pathway. Thus, the findings provide evidence for the potential application of oridonin in depression therapy.*

Keywords: *Oridonin, Depression, Chronic unpredictable mild stress, TXNIP/NLRP3 signaling pathway*

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INTRODUCTION

Depression, a condition of mental disturbance, represents a series of presentations characterized by physical, emotional, and related cognitive disorders [1]. The World Health Organization (WHO) has ranked depression as the fourth prime contributor of worldwide disease burden by 2020 [2]. Chronic stress is a primary factor of depression, the cause of which is immune disorders and inflammation [3]. Studies have demonstrated that inflammation is related

to the complex process of depression [4]. Although many antidepressants improve depressive symptoms, they are associated with undesirable side effects, including suicidal tendencies, sexual dysfunction, and sleep disturbances [5]. Therefore, studies on antidepressants are urgent.

Thioredoxin-interacting protein (TXNIP), which detaches from thioredoxin (TRX) and binds to nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), participates in inflammation-related diseases [6]. TXNIP is also a negative regulator of TRX and is considered a stress protein and key regulator of the inflammatory response. Increasing evidence has shown that the elevated expression of TXNIP is related to chronic stress and depression, and TXNIP deficiency inhibits inflammatory responses [7].

Oridonin (Figure 1A), a natural bioactive tetracycline diterpenoid, is a flavonoid compound isolated from *Rabdosia rubescens* that was first identified as an antitumor compound [8]. It has attracted increased attention because of its various pharmacological effects, including anti-inflammatory and anti-oxidant activities [9]. For example, oridonin binds NLRP3, a central component of the inflammasome, and further inhibits NLRP3 inflammasome activation [10]. *In vitro*, oridonin inhibits the expression of inducible nitric oxide synthase and prostaglandin-endoperoxide synthase 2 by inhibiting nuclear factor kappa B DNA binding activity [11]. Additionally, oridonin exerts a protective effect on the central nervous system. Zhang *et al*. found that oridonin significantly attenuated β-amyloid deposition and microglial activation in the mouse brain [12]. However, few studies have reported the effects of oridonin on depressive behaviors caused by chronic stress, and the relevant molecular mechanism requires further study. The present study aimed to investigate the effects of oridonin on chronic unpredictable mild stress (CUMS)-induced depressive-like behaviors and determine the mechanism.

EXPERIMENTAL

Animals and treatment

Twenty-four mice (C57BL/6J, approximately 6-weeks old) were purchased from Beijing Vital River Laboratory Animal Technology. CUMS was established referring to a previously reported method s[3]. The stress stimuli lasted 6 weeks. The stress stimuli included feed/water deprivation (24 h), night lighting, tilting of the cage (45º), a wet cage (200 mL of water/100 g of padding material), exposure to the external environment, an inverted light/dark cycle, tail clamping (1 min), exposure in an empty bottle, strobe lighting, and shocking. The animals were divided into four groups: control, CUMS, CUMS + Oridonin (Ord, 10 mg/kg), and CUMS + Fluoxetine (Flu, 10 mg/kg). All the experiments were in accordance with the Guide for the Care and Use of Laboratory Animals [13] and were approved by the Ethics Committee of Whenzhou No. 7 People's Hospital (approval no. 2018117).

Sucrose preference test

The sucrose preference test (SPT) was performed on day 40. The animals had undergone adaptive training for 24 h with two bottles of 1 % sucrose, and then one bottle was replaced with pure water for 24 h. After 24 h of feed and water deprivation, each animal was given a bottle of pure water and 200 mL of another bottle of 1 % sucrose solution. The consumption of pure water and sucrose was recorded after 1 h and again after 12 h. To avoid the objective factors, the distance between the two bottles was kept unchanged and the positions were switched.

$$\text{Sucrose preference (\%)} = \frac{\text{Sucrose intake (g)}}{\text{Sucrose intake (water intake)}} \times 100.$$

Forced swimming test

The forced swimming test **(**FST) apparatus was a cylinder filled with 10-cm-deep water (23 ± 2 °C). At the end of the CUMS protocol, a pretest was performed for 15 min. For the pretest, the animals were allowed to swim individually for 15 min. After that, the animals were removed from the water, dried, and placed in a warm enclosure. After 24 h, the procedure was repeated. The top view was recorded. Animals that floated without swimming were judged to be immobile. The immobility time was measured over 4 min after 6 min of adaptable swimming.

Tail suspension test

In the tail suspension test (TST), immobility was induced by suspending the mice by the tail. The animals were individually suspended from the tip of the tail 50 cm above the ground for 6 minutes. The mice remaining completely motionless were considered immobile.

Open field test

Locomotor activity was evaluated using the open field test (OFT). The apparatus was a black square cage with the floor divided into 12 equal squares. Animals freely explored the

environment for 4 min, with 30 sec to adapt to the environment. The numbers of square crossing, grooming, and rearing events were recorded by a camera above the field.

Determination of cytokines in the hippocampus

The hippocampus of the mice was removed and homogenized in normal saline. The total protein was quantified using the bicinchoninic acid (BCA) method. The levels of tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein-1 (MCP-1) were analyzed by the enzyme-linked immunosorbent assay (ELISA).

Western blotting

Cells were lysed with RIPA lysis buffer, and total protein concentrations were quantified using the BCA method. Equal amounts of cell lysates were separated using SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After blocking, each membrane was incubated with antibodies (1:1000 dilution) overnight at 4 °C and then incubated with the secondary antibody. The antibodies were as follows: anti-TXNIP (CST; #14715), anti-NLRP3 (CST; #13158), cleaved-caspase 1 (CST; #89332), and HRP-linked anti-rabbit IgG (CST; #7074). Finally, protein expression was visualized using an ECL system and analyzed by ImageJ software.

Statistical analysis

All the data were shown as means ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test as the post hoc test. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of oridonin on the motivational behavior of CUMS mice

The effects of oridonin (chemical structure shown in Figure 1 A) on motivational behavior were analyzed using the SPT, TST, and FST. Anhedonia is a major characteristic of depression, measured in this study by reduced consumption of sucrose. The sucrose consumption of CUMS-treated mice was significantly decreased compared with that of the controls (Figure 1 B; $p < 0.01$), and oridonin (Ord) and fluoxetine (Flu) treatment attenuated the decreasing consumption ($p < 0.01$ vs. CUMS). The immobility time in the FST (Figure 1 C) and TST (Figure 1 D) was increased in CUMS mice ($p < 0.01$ vs. control), and oridonin and fluoxetine reduced the immobility time compared with mice treated with CUMS alone ($p < 0.01$). Fluoxetine treatment was more effective than oridonin treatment in motivational behavior improvement.

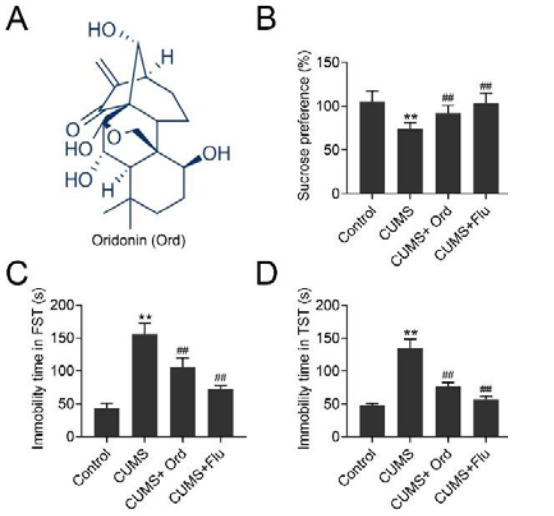

Figure 1: Effect of oridonin (Ord) and fluoxetine (Flu) on the motivational behavior of chronic unpredictable mild stress (CUMS) mice. (A) Chemical structure of oridonin. The sucrose preference (B), immobility time in the forced swimming test (FST) (C) and tail suspension test (TST) (D) of CUMS and Ord-treated mice; n = 6, $^{**}p < 0.01$ vs. Control; $^{\#\#}p < 0.01$ vs. CUMS

Oridonin affects the locomotor activity of CUMS-treated mice

Spontaneous locomotor activities were measured in OFT. In the CUMS-treated group, the number of crossing, rearing, and grooming events decreased significantly ($p < 0.01$ vs. control). Oridonin and fluoxetine reversed the effects of CUMS treatment ($p < 0.01$ vs. CUMS), and the number of events in the fluoxetine group was closer to that of control (Figure 2).

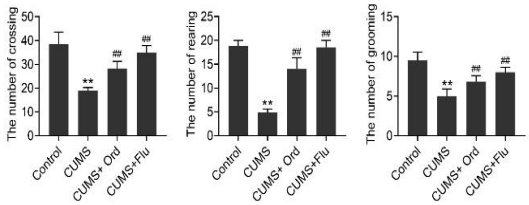

Figure 2: Oridonin (Ord) and fluoxetine (Flu) affect the locomotor activity of (chronic unpredictable mild stress) CUMS-treated mice. Number of crossing, rearing, and grooming events in the open field test; n = 6, $^{**}p < 0.01$ vs. Control; $^{\#\#}p < 0.01$ vs. CUMS

Oridonin decreases the production of cytokines in the hippocampus

The levels of TNFα, IL-1β, IL-6, and MCP-1 were dramatically increased in the CUMS group (p < 0.01 vs. control), and oridonin and fluoxetine significantly inhibited the CUMS-induced increases of these cytokines in the hippocampus (p < 0.01 vs. CUMS) (Figure 3), indicating that oridonin attenuated the excess inflammatory responses induced by CUMS.

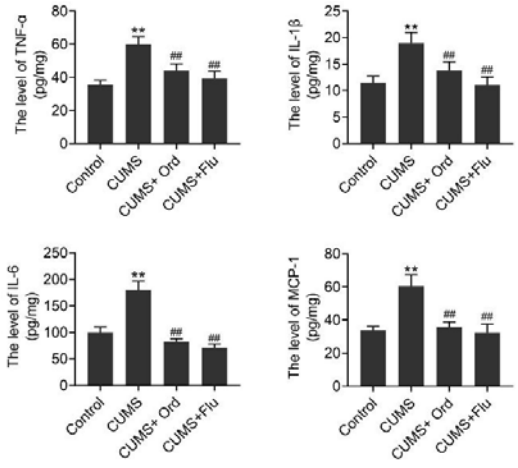

Figure 3: Oridonin (Ord) and fluoxetine (Flu) decrease the production of cytokines in the hippocampus. The levels of tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein-1 (MCP-1) in the hippocampus were analyzed by enzyme-linked immunosorbent assay; n = 6, $^{**}p$ < 0.01 vs. Control; $^{\#\#}p$ < 0.01 vs. CUMS

Effect of oridonin on the expression of TXNIP/NLRP3 pathway-related proteins

To investigate the effects of oridonin on inflammatory responses, TXNIP/NLRP3 pathway-related proteins were analyzed by western blot. We previously showed that, in CUMS-treated mice, TXNIP/NLRP3 is activated, and the expression of TXNIP, NLRP3, and cleaved caspase-1 increased (Figure 4). As expected, oridonin treatment significantly decreased the levels of TXNIP/NLRP3 proteins (p < 0.01 vs. CUMS).

DISCUSSION

Chronic stress is a major risk factor for depression, a chronic and recurrent syndrome of mood disorder. CUMS is a model that mimics stress-induced depression and has been widely used in preclinical research [3].

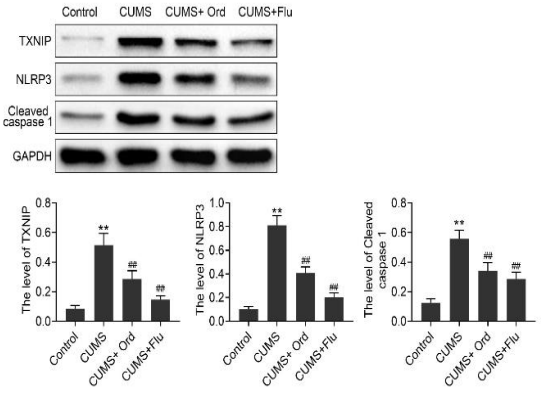

Figure 4: Effect of oridonin (Ord) and fluoxetine (Flu) on the expression of TXNIP/NLRP3 pathway-related proteins. Expression of thioredoxin-interacting protein (TXNIP), nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), and cleaved caspase 1 in the hippocampus was analyzed by western blot; $^{**}p$ < 0.01 vs. Control; $^{\#\#}p$ < 0.01 vs. CUMS

Previous studies have confirmed that the CUMS model shows depressive behavior, such as decreased sucrose consumption [14]. In the present study, after the CUMS procedure (45 days), mice displayed depressive-like behaviors and cognitive defects, including less sucrose consumption, increased immobility time, and a reduced number of crossing, rearing, and grooming events in the OFT. However, oridonin administration reversed these depressive-like behaviors in CUMS mice, similar to the positive drug (fluoxetine).

Depression is associated with inflammatory responses [15], and depressed patients demonstrate abnormal production of pro-inflammatory cytokines [4]. Studies have also shown that pro-inflammation cytokines play a key role in the progression of inflammation, which induces depressive behaviors [16]. Many studies have shown that attenuating inflammation in the hippocampus significantly alleviates depressive behaviors [17]. For example, baicalein, a flavonoid glycoside isolated from *Radix Scutellariae*, ameliorates neuro-inflammation and depressive behaviors by inhibiting the phosphoinositide 3-kinase/AKT pathway [18]. Oridonin has garnered increased attention because of its extensive biological activities, including anti-inflammatory effects. Xu *et al.* reviewed the effects of oridonin and its analogs on inflammation and highlighted its role as a therapeutic candidate in neuroprotection [19]. In the present study, the levels of pro-inflammation cytokines in the hippocampus were increased significantly after CUMS treatment and were all inhibited by oridonin, suggesting a potential anti-inflammatory effect of oridonin in depression.

TXNIP, an inhibitor of TRX, is now known to be a critical regulator of NLRP3 inflammasome activation [20]. TXNIP overexpression activates inflammatory pathways via TRX-mediated inflammation inhibition or by directly activating the NLRP3 inflammasome [21]. Previous studies have suggested that TXNIP dissociates from TRX and binds to NLRP3, a multiple protein complex comprising NLRP3, apoptosis-associated speck-like protein, and caspase-1. The assembly of this complex results in caspase-1 activation, further producing mature IL-1β, which plays a central role in inflammation [22]. Thus, in the present study, activation of the TXNIP/NLRP3 signaling pathway was analyzed. Oridonin inhibited the expression of TXNIP, NLRP3, and cleaved caspase-1 induced by CUMS, suggesting a possible mechanism for the anti-depression effects of oridonin. A similar effect of oridonin was found in mice with myocardial ischemia/reperfusion (I/R) injury [10]. Oridonin alleviated I/R induced myocardial injury by inhibiting oxidative stress and the NLRP3 inflammasome pathway [10].

CONCLUSION

The findings of this study suggest that oridonin ameliorates depressive-like behaviors of CUMS mice partly via TXNIP/NLRP3 signaling pathway, and thus, provide evidence for the potential application of oridonin in depression therapy.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lina Wang and Ziye Huang designed the study and supervised the data collection. Jing Ping analyzed and interpreted the data. XingYan Liu, Ziyao Cai, and Chenghao Dai prepared the manuscript for publication and reviewed the draft of the manuscript. All the authors read and approved the manuscript.

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