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Original Research Article

Crassifoside H ameliorates depressant behavior in chronic unpredictable mild stress rats by improving HPA axis dysfunction and inhibiting inflammation in hippocampus

Huina Li[1], Kefan Wu[1], Yue Zhang[2], Ning Li[2], Kaijin Wang[1]*
[1]School of Life Sciences, Anhui University, [2]School of Pharmacy, Anhui Medical University, Hefei, China

For correspondence:* ***Email:* *wkjahla@163.com;* ***Tel/Fax:*** *+86-551-63861297*

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Abstract

Purpose: *To investigate the antidepressant mechanism of action of Crassifoside H (CH) from the rhizomes of Curculigo glabrescens (Hypoxidaceae) in chronic unpredictable mild stress (CUMS)-induced rats.*
Methods: *CUMS-induced rat depressant model was established. Behavioral tests, viz, sucrose preference test (SPT), open field test (OFT) and forced swimming test (FST) were applied to assess the antidepressant effect of CH. Enzyme linked immunosorbent assay (ELISA) was used to assess the levels of corticosterone (CORT), TNF-α and IL-1β in serum. Protein expressions of TNF-α, IL-1β and NLRP3 in rat hippocampus were determined by Western blot.*
Results: *Crassifoside H significantly ameliorated CUMS-induced depressant-like behavior as the serum CORT level of CUMS rats. CH remarkably decreased TNF-α and IL-1β levels in serum and hippocampus of CUMS rats. Moreover, Crassifoside H significantly inhibited NLRP3 activation in hippocampus.*
Conclusion: *The findings demonstrate that Crassifoside H has antidepressant effect on CUMS rats. The mechanism of action of CH may be at least partly due to the improvement of hypothalamic-pituitary-adrenal (HPA) axis dysfunction by decreasing serum CORT. These findings suggest that Crassifoside H has a therapeutic potential for the management of depression.*

Keywords: *Crassifoside H, Antidepression, Curculigo glabrescens, Hypoxidaceae, Hypothalamic-pituitary-adrenal axis, Inflammation, Corticosterone*

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INTRODUCTION

Depression is a common psychiatric disease with high prevalence and heavily influences patient's health and life, and imposes huge burden on individuals, families and society [1]. Malhi and Mann predicted that depression would be the first leading disease worldwide by 2030 [2]. Owing to the complexity of depression, current antidepressants are far from satisfaction due to a variety of adverse effects, and approximately one third of the major depressive disorder (MDD) patients have no respond to the existing medications. Therefore, it is important to find novel and effective agents for this disease.

Increasing evidence suggests that depression is a neuroinflammatory disorder, and several inflammation-related cytokines are implicated in the development of this disease [3]. The inflammatory cytokines, i.e. interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), are high level in depressed patients [4]. Moreover, inflammatory cytokines are involved in the dysfunction of hypothalamic-pituitary-adrenal (HPA) axis, cerebral noradrenergic systems and brain serotonergic systems in depressed patients [5]. IL-1β is the first reported inflammatory cytokine that cause the hyper-activation of HPA axis in depression [6]. TNF-α is another important inflammatory cytokine involved in depression by influencing activity of HPA axis and serotonin metabolism. Some antidepressants displayed the ability to restore the disorders of HPA axis and TNF-α [7].

Inflammasome NLRP3 is an intracellular protein complex consists of a nod-like receptor protein 3, adaptor protein ASC and procaspase-1. The activation of NLRP3 inflammasome in cell promotes the caspase-1 maturation and downstream IL-1β release [8]. Normal NLRP3 activation is necessary for host immune defense. However, over-activation may cause excessive generation of IL-1β, which is implicated in the pathological processes of various inflammation-related diseases like depression [9]. Growing studies have recorded that NLRP3 is highly activated in MDD patients and stress-treated model animals [10, 11]. NLRP3 inflammasome have been recognized as a promising therapeutic target for depression [12].

Herbal medicines and natural products with antidepressant effect are considered much safer in the treatment of depression than chemical antidepressants [13]. Crassifoside H (CH) is a norlignan compound obtained from the rhizome of *Curculigo glabrescens* (Hypoxidaceae) [14]. Recently, CH was found to exert antidepressant-like behavior by enhancing the expression of 5-HT_1A receptor and promoting the activation of BDNF-ERK signaling pathway [15].

As mentioned above, HPA axis hyperactivation and neuro-inflammation are important in the development of depression, and whether the antidepressant-like mechanism of CH is associated with them still unclear. In order to further explore the possible active mechanism of CH, rat CUMS depression model was established in this study, the concentration of CORT, TNF-α and IL-1β in serum; as well as protein expression of TNF-α, IL-1β and NLRP3 in rat hippocampus were evaluated.

EXPERIMENTAL

Drugs and reagents

Crassifoside H (CH, 99.0%) was prepared in the lab from the rhizomes of *C. glabrescens* (Figure 1). Fluoxetine hydrochloride was from Eli Lilly Pharmaceuticals (Suzhou, China). Corticosterone (CORT) was bought from Shanghai Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). Fetal bovine serum was purchased from Gibco BRL (Grand Island, NY, USA); CORT assay kit was purchased from Enzo life sciences, Inc. (USA), while TNF-α and IL-1β assay kits were from Neobioscience Technology Company (China).

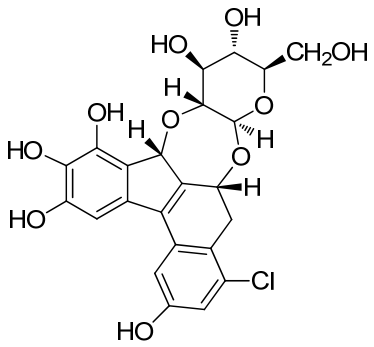

Figure 1: Chemical structure of crassifoside H

Animal grouping

Forty-eight adult male Sprague-Dawley rats, weighing 200-220 g, were provided by Experimental Animal Center of Anhui Medical University. Animals were accommodated in cages (43 × 31 × 19 cm) under controlled conditions as following: room temperature (21 ± 1 °C), 12 h light/12 h dark cycle, *ad libitum* access to feed and water. After 7 days of adaptation, rats were divided into 6 groups (n=8) at random: control group, chronic unpredictable mild stress (CUMS) group, three CH groups (i.e. CUMS + crassifoside H; 2, 4, and 8 mg/kg.d), and fluoxetine group (CUMS + fluoxetine; 2.5 mg/kg.d). Animal experimental protocols followed the guideline of “Principles of Laboratory Animal Care” [16] and were approved by Animal Ethics Committee of (no. AEC 2019-20) Anhui University, China.

CUMS procedures and drug administration

The procedures of CUMS were conducted for 4 weeks as follows. In brief, the stress including: (1) 12-h deprivation of sleep; (2) electric shock; (3) 2-h noise disturbance; (4) 24-h deprivation of feed and water; (5) 5-min swimming in warm

water (40 °C); (6) tail pinch for 2-min; (7) whole-day wet bedding; (8) 45° tilted cage for 24 h; (9) 5-min swimming in cold water (4-6 °C); (10) overnight lighting. The experiments were applied between 8:00 a.m. and 11:00 p.m. (except 12h and 24h stress) once daily. All stress were performed randomly to ensure the unpredictability. The CH or fluoxetine were orally administered from day 22 to day 28, and the control and CUMS group were given 0.5% CMC-Na.

Body weight

Body weight was recorded at the beginning of the CUMS procedures and thereafter at weekly interval until the end of the CUMS experiment 4 weeks later.

Behavioral tests

Animal behavioral experiments were carried out in a soundproofed laboratory as the schedule below. SPT was conducted on day 29, followed by OFT on day 30, and FST on day 31. All the tests were carried out from 8:30 a.m. to 14:30 p.m.

SPT was performed on day 29. After 12-h deprivation of water, the rats were let to drink from two bottles for 4 h; one bottle of water, and the other 1% sucrose solution. The consumptions of water and sucrose solution were recorded. The sucrose preference index was obtained as formula: consumption of sucrose solution/total liquid intake.

OFT was conducted in a black wooden box (100 × 100 × 30 cm). The floor of the arena was divided into 16 equal squares using white bold lines. The animals were put in the corner of the box with the liberty to explore freely. The numbers of rearing, crossing and grooming were recorded for 5 min. The apparatus was cleaned with 90% ethanol and dried before next test to remove the smell from former animal.

In FST, rats were separately put in a glass vessel (60 cm tall, 25 cm diameter) filled with water with depth of 30 cm to swim 5 min. During experiment, the temperature of water was kept at 24-25 °C. The immobility time was recorded when rat kept floating without any active struggles.

Sample collection

The day after FST, the animals were sacrificed immediately under deeply anesthesia. The blood was collected from abdominal aorta and the serum was obtained by centrifugation at 4°C, 3000 r/min for 15 min. The brain was rapidly excised from head in ice-bath and the hippocampus was quickly isolated.

Serum CORT, TNF-α and IL-1β level determination

Levels of CORT, TNF-α and IL-1β in serum was measured using ELISA kits under the guide of product instructions.

Western blot analysis

The hippocampus samples were homogenized in ice-cold protein lysis buffer, then removed debris by centrifugation (4 °C, 12,000 r/min, 30 min). Bradford method was used to determine the protein concentrations of the supernatants. The protein lysates were separated on 10% SDS-PAGE gel and transferred to a PVDF membrane, blocked with 5% skim-fat milk in TBST buffer for 1 h, and then overnight incubated with primary antibodies targeting TNF-α (1:1000; ImmunoWay Biotechnology Company, Suzhou, China), IL-1β (1:1000; Santa Cruz Biotechnology, Shanghai, Co. Ltd., China), NLRP3 (1:1,000; ImmunoWay Biotechnology Company, Suzhou, China), or β-actin (1:1000; ZSGB-BIO, Beijin, China) at 4 °C, followed by HRP-conjugated secondary antibodies (1:10000) for 1h. Blots were probed by Easysee ECL Western Blot Kit (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

The statistical analysis was conducted using SPSS 17.0. The values were presented as means ± SD, and analyzed using one-way analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

CH administration improved depressant-like behavior of CUMS-treated rats

The changes of CH on rat body weight were presented in Figure 2A. After 4-week stress, rats in control group gained more body weight than that of rats in CUMS and drug-treated groups ($p < 0.01$), no significant differences between CUMS group and drug-treated groups ($p< 0.05$). These findings indicate that the CUMS had a negative effect on the rat body weight gain.

For human, anhedonia is the central symptoms of depression. The sucrose preference index in SPT is a parameter designed in rat model to describe this mood state [17]. Figure 2B revealed

that the CUMS rats significantly decreased the sucrose solution consumption compared with the rats of the control group ($p < 0.01$), while CH at dose of 2, 4, 8 mg/kg or fluoxetine at 2.5 mg/kg greatly reversed this decline when compared that of the CUMS animals ($p < 0.05$), indicating that CH significantly improve the anhedonic condition of CUMS rats.

FST is a canonical experiment applied to estimate despair and helpless in depression [18]. In Figure 2C, CUMS rats showed longer immobility time than control group ($p < 0.01$), while rats received CH treatment spent more time in swimming than CUMS group ($p < 0.05$), which suggest that CH administration alleviate the despair behavior of depression.

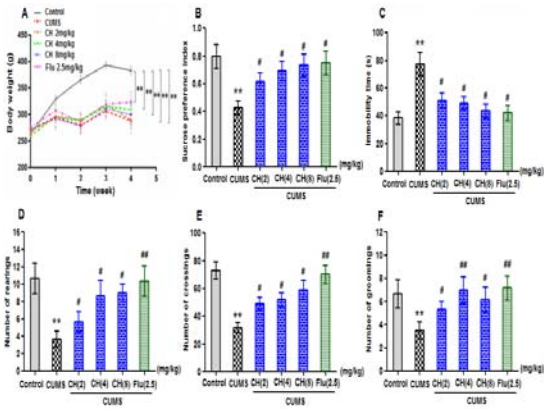

Figure 2: CH improved depressant-like behavior of CUMS rats. A: body weight change; B: sucrose preference index in SPT; C: immobility time in FST; D-F: number of rearing, crossing and grooming in OFT. Data are expressed as mean ± SD (n = 8), $^{**}p < 0.01$ vs control group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ and vs CUMS group

OFT is common experiment to estimate locomotor and exploratory activity of animal under a novel environment [19]. As shown in Figure 3D-3F, CUMS rats significantly decreased their actions of rearing, crossing and grooming. CH effectively enhanced the locomotor and exploratory activities of CUMS-treated rats, indicated by the higher frequency of rearing, crossing and grooming.

CH administration alleviated HPA axis dysfunction

High level of CORT is the marker of hyperactivity of HPA axis. As shown in Figure 3, after 4-week CUMS procedures, the level of serum CORT in CUMS group was 610.5 ng/mL, which was more than twice as that of the control group (235.2 ng/mL). CH and fluoxetine significantly decreased CORT level in serum compared with CUMS group. These results suggested that CH administration alleviated this abnormality in CUMS rats.

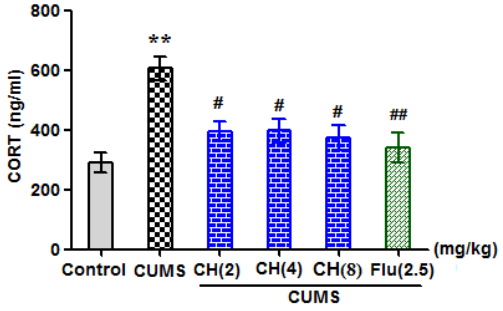

Figure 3: CH decreased serum CORT in CUMS rats. Data are expressed as mean ± SD (n = 8), $^{**}p < 0.01$ vs control group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.05$ vs CUMS group

CH administration decreased serum TNF-α and IL-1β of CUMS rats

Figure 4 displayed that the levels of TNF-α and IL-1β in serum of CUMS rats were much higher than those in control group ($p < 0.01$). While CH and fluoxetine significantly reversed this increasing tendency.

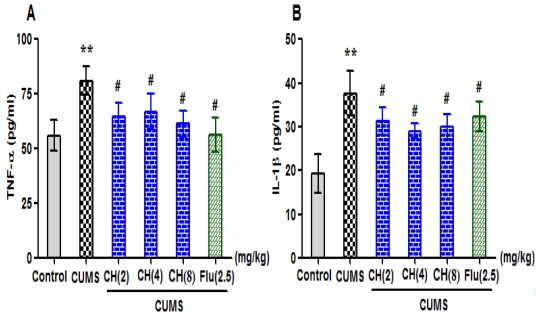

Figure 4: CH deceased serum TNF-α (A) and IL-1β in CUMS rats. Data are expressed as mean ± SD (n = 8); $^{**}p < 0.01$ vs control group, $^{\#}p < 0.05$ vs CUMS group

CH administration inhibited protein expression of TNF-α, IL-1β and NLRP3 in CUMS rat hippocampus

As shown in Figure 5, the expressions of TNF-α, IL-1β and NLRP3 in hippocampus were significantly increased in CUMS rats when compared to control group (TNF-α and IL-1β: $p < 0.05$; NLRP3: $p < 0.01$), while CH and fluoxetine remarkably decreased expression of TNF-α, IL-1β and NLRP3 in hippocampus of CUMS-treated rats. In addition, the synchronous changes in NLRP3 and IL-1β further suggest that activation of NLRP3 and the downstream release of IL-1β were involved in CUMS-induced inflammatory process.

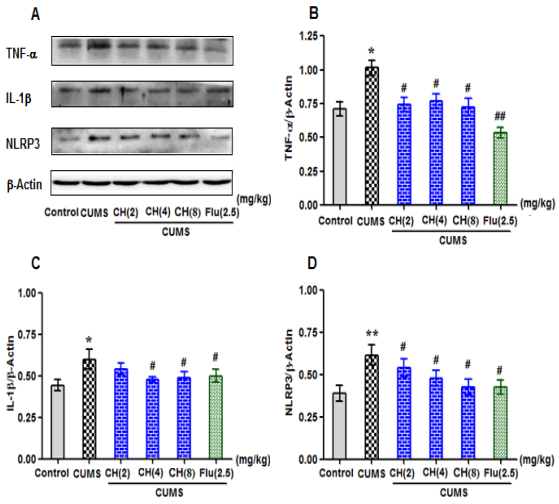

Figure 5: Effect of CH treatment on the protein expressions of TNF-α, IL-1β and NLRP3 in hippocampus of CUMS rats. A: Western blot analysis of TNF-α, IL-1β and NLRP3. B-D: Relative ratios of TNF-α, IL-1β and NLRP3. Data are expressed as mean ± SD (n=8); $^{*}p<0.05$ and $^{**}p < 0.01$ vs control group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs CUMS group

DISCUSSION

CUMS is a classical and reliable model applied to discover the antidepressants and related mechanisms. In the experiment, animals are under a series of unpredictable stress, which imitated different stressful events in human life and induced depressive-like behavior and mood states (e.g., anhedonia and despair, etc.) in MDD patients [20]. In the present study, characteristic depressive-like behavior was observed in the experiments including SPT, OFT and FST; while CH administration significantly alleviated the behavioral abnormality in CUMS rats.

Hyperactivation of HPA axis is an important pathological event in MDD [21]. External stress leads to the elevation of the baseline level of glucocorticoids (GCs; cortisol for man and CORT for rats). Hypothalamus, the key target organ of GCs, is the vital center that controls HPA axis. Prolonged high level of CORT causes the inflammation in hippocampus, induce atrophy of pyramidal neurons, and finally result in depressive symptoms [22]. In addition, the hormones alteration is reported closely linked with the HPA axis abnormality [23]. Corticotropin-releasing hormone (*CRH*) leads to the release of *adrenocorticotropic hormone* (ACTH) in anterior pituitary gland, and in turn ACTH promotes the generation of GCs in cortex [24]. In current study, high serum CORT were observed in CUMS group, while CH administration effectively reversed this change in CUMS-induced rats, suggesting that CH alleviates the dysfunction of HPA axis.

Growing studies have revealed that the inflammation is implicated in the development of depression [3]. Excessive inflammations were observed in MDD patients and model animal of depression [25]. Internal and external stress may trigger immune and inflammation processes, lead to the release of inflammatory cytokines, and cause inflammation in important brain regions including hippocampus and hypothalamus [26]. Fluoxetine and paroxetine were reported to exert antidepressant effect by inhibited the inflammation through reducing related cytokines in the brain [27]. In present study, CUMS not only elevated TNF-α and IL-1β in serum, but also up-regulated them in hippocampus, suggesting the neuro-inflammation in CUMS model rats. However, CH administration effectively decreased the them both in serum and hippocampus. These findings suggested that the antidepressant effect of CH might be due to its inhibitory ability on TNF-α and IL-1β.

Besides inflammatory cytokines, NLRP3 inflammasome also contributes greatly to the neuroinflammation in depression [9]. Hyperactivation of NLRP3 leads to the over-generation of IL-1β, which is a key mediator participated in the inflammatory process in brain [11]. Increasing research have documented that NLRP3 activation performs an important role in various central nervous system (CNS) diseases include depression, and NLRP3 inhibition has demonstrated the potential for depression treatment [12]. In current study, NLRP3 protein production increased significantly accompanied with the release of IL-1β in CUMS rat hippocampus, suggesting that CUMS induced the activation of NLRP3 in and promoted the release of IL-1β which aggravated the neuroinflammation in rat hippocampus. However, CH significantly inhibited the inflammation by blocked the activation of NLRP3 and decreased IL-1β in hippocampus in CUMS-treated rats. These results indicate that the inhibition of NLRP3 activation might have important contribution to the antidepressant effect of CH.

CONCLUSION

The present study demonstrates that CH exerts antidepressant effect on CUMS rats. The antidepressant mechanism of action of CH may be at least partly due to the improvement of dysfunction of HPA axis by decreasing serum CORT level. Moreover, the suppression of inflammation by decreasing TNF-α and IL-1β, as well as inhibiting the activation of NLRP3 in

hippocampus might be responsible for the antidepressant effect. These findings suggest that Crassifoside H is a potential therapeutic agent for the management of depression.

DECLARATIONS

Acknowledgement

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Conflict of interest

The authors declare that no conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. HN Li and Y Zhang performed the experiments, KF Wu conducted the statistical analysis, while N Li and KJ Wang designed, and supervised the experiments and wrote the manuscript.

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