



Safflower extract improves depression in mice by inhibiting the TLR4-NLRP3 inflammation signaling pathway

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Background: Safflower extract (SE) improves depression in mice by inhibiting the TLR4-NLRP3 inflammatory signaling pathway.

Methods: Chronic unpredictable mild stress (CUMS) was used to establish a mouse model of depression. A total of 60 adult male ICR mice were randomly divided into 6 groups: control group, depression group (CUMS only), SE (10 mg/kg) + depression group (CUMS+SE, 10 mg/kg), SE (30 mg/kg) + depression group (CUMS+SE, 30 mg/kg), Cli-095 + depression group (CUMS+Cli-95), and fluoxetine hydrochloride (FLU) + depression group (CUMS+FLU). We assessed the depressive behaviors of these mice using the sucrose preference test (SPT), the open field test (OFT), the forced swim test (FST), and the tail suspension test (TST). We measured the expression levels of SOD, MDA, GSH-Px, 5-HT, NE, TNF- α , IL-1 β , and IL-6 using ELISA kits. Western blot was used to determine the relative expression levels of TLR4, p38, NF- κ B, NLRP3, and caspase-1.

Results: SE significantly improved the results of the SPT, OFT, FST, and TST. SE also increased the expression levels of 5-HT and NE in the prefrontal cortex while decreased the expression levels of TNF- α , IL-1 β , and IL-6 compared with CUMS. SE (10 or 30 mg/kg) or FLU (10 mg/kg) significantly inhibited the expression of TLR4 and p-p38 induced by CUMS. SE significantly inhibited the expression of p-NF- κ B in the prefrontal cortex and hippocampus induced by CUMS. The decrease of NLRP3 and caspase-1 were obviously reversed after administration of SE (10 or 30 mg/kg) or FLU (10 mg/kg).

Conclusions: The results showed that SE has potential antidepressant effects in CUMS mice, and its underlying mechanism may be related to its effects on inflammation and the TLR4-NF- κ B-NLRP3 signaling pathway in the brain.

Keywords: Safflower extract (SE); TLR4-NLRP3; depression

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Introduction

Depression is a common and serious mental disorder affecting 16% of the world's population. It is characterized by general depression, loss of interest in usual activities, and is accompanied by symptoms such as physical discomfort (1). Nowadays, there are many antidepressants, including selective serotonin reuptake inhibitors (SSRIs), monoamine

oxidase inhibitors (MAOIs), and tricyclic antidepressants (TCA). Emerging evidence has shown that depression is associated with inflammation (2). Clinical studies have suggested that compared with healthy people, patients suffering from depression have higher circulating levels of pro-inflammatory cytokines (3). Animal experiments have also demonstrated that depressed mice produced

large amounts of cytokines including interleukin-6 (IL-6), interleukin-1 β (IL-1 β), and tumor necrosis factor- α (TNF- α) in the serum and brain tissue (4). In addition, some meta-analyses found that pro-inflammatory cytokines are positively correlated with depression. However, the mechanism involved has not yet been clearly investigated. IL-1 β is an important neuroendocrine mediator, and its production and secretion are mainly regulated by the inflammasome. The nucleotide-binding oligomerization domain-like receptor family NLRP3 inflammasome is a cytoplasmic receptor containing NLRP3, ASC, and caspase-1 (5). Therefore, regulating the TLR4-NLRP3 signaling pathway and further inhibiting neuroinflammation may be a treatment strategy to improve depression. Safflower (*Carthamus tinctorius* L.) has long been a hot topic of research due to its diverse chemical components as well as extensive pharmacological effects. More and more studies have shown that safflower extract (SE) could inhibit inflammation and oxidation, protect the heart, and enhance immune function (6). Studies have shown that spermidine in safflower can alleviate depression symptoms, but the underlying mechanism is not yet clear (7). Whether SE can inhibit inflammation by inhibiting the TLR4 signaling pathway in depression remains unclear. In this study, we speculated that SE could treat depression through the inhibition of TLR4 activation and NLRP3 downregulation. To test this hypothesis, we established a mouse model of depression to investigate the effect of SE on the expression of the inflammatory cytokines TLR4 and NLRP3.

We present the following article in accordance with the ARRIVE reporting checklist (available at <https://dx.doi.org/10.21037/apm-21-1728>).

Methods

Animals

A total of 60 adult male ICR mice (18–22 g) were purchased from the Laboratory Animal Center, Zhongshan School of Medicine, Sun Yat-sen University. Mice were placed in cages alone for about 1 week to let them adapt to the environment under standard housing conditions: 12 h light/12 h dark cycle; abundant food and water supply; temperature maintained at 22 \pm 2 °C and humidity of 50% \pm 10%. All animal experiments were approved by the Animal Experiment Ethics Committee (AEEC) of Zhongshan School of Medicine and were in compliance

with the Guide for the Care and Use of Laboratory Animals.

Medications and treatment

Fluoxetine hydrochloride (FLU) was purchased from Changzhou Siyao Pharmaceutical Co., Ltd. (Changzhou, China); safflower extract was purchased from Nanning Shengyuan Chinese Medicine Co., Ltd. (Lot No. 140901, Nanning, China) and decocted in water to prepare SE solution. Cli-095 (TLR4 specific inhibitor) was purchased from Invivo Gen (San Diego, California, USA) and dissolved in 1% dimethyl sulfoxide (DMSO). Depression was induced with chronic unpredictable mild stress (CUMS). The mice were randomly assigned into 6 groups: control group, depression group (CUMS only), SE (10 mg/kg) + depression group (CUMS+SE, 10 mg/kg), SE (30 mg/kg) + depression group (CUMS+SE, 30 mg/kg), Cli-095 + depression group (CUMS+Cli-95), and FLU + depression group (CUMS+FLU). Both the control group and the CUMS group were given intragastric administration of medication dissolved in sterile 0.9% normal saline. The SE treatment group received different concentrations of SE (10 and 30 mg/kg). The concentration of Cli-095 for the Cli-095 treatment group was 3 mg/kg. The concentration of FLU for the FLU treatment group was 10 mg/kg. Treatments were given once daily for 4 consecutive weeks. In order to determine whether SE can alleviate depression, we used CUMS to establish a mouse model of depression. After 1 week of adaptation to the environment, the mice were exposed to CUMS for 2 weeks (week 1 and 2). Following the exposure, these mice were administered with either SE, Cli-095, or FLU for 4 weeks (week 3–6). CUMS continued during treatment (week 3–6).

Depression model

After 1 week of adaptation to the new environment, the mice in the control group were kept in a separate room and the other 5 groups were modeled for depression. These animals were exposed to stress every week, including 12 h without food, 5 min cold swimming, 10 min cage shaking, 30 min behavior restraint, 5 min tail pinch, 8 h light/dark reversal, and 12 h cage tilting with a 45° angle. We applied stressors continuously during the day and night individually. In order to prevent the habituation of mice to the stressor

and ensure the unpredictability of stressors, all stressors were randomly arranged within a week and repeated throughout the experiment.

Sucrose preference test (SPT)

In the first 24 h, 2 bottles (same bottle) of 1% (w/v) sucrose solution (SS) were placed in each cage in order to train mice to adapt to the SS. After 24 h of adaptation, we replaced 1 bottle of SS with cooled boiled-water (CBW) in the same bottle. The positions of the water bottle and the SS bottle were exchanged every 12 h to avoid any influence caused by the bottle location. When these mice adapted to the SS, we deprived them of water and food for 12 h. At 24 h later, the volumes of SS and CBW consumed were calculated. Sucrose preference (SP) = volume of SS consumption/(volume of CBW consumption and volume of SS consumption) ×100%.

Open field test (OFT)

Locomotor activity was measured by the OFT. The testing field consisted of a lighted glass-box (100 cm long, 100 cm wide, and 50 cm high) and a real time monitoring system. The test subject was initially placed right in the bottom center of the box, and was allowed to move freely in the box. Then, the total movement distance during 60 min was recorded automatically.

Forced swim test (FST)

The test subject was put in an open cylindrical glass water tank (diameter 20 cm, height 40 cm) filled with water of 25 cm depth at 22±1 °C. The environment around the tank was kept quiet when the test was conducted. The mice were placed in the cylinder individually for 6 min, and immobility time was recorded during the last 4 min of the test. The mouse was considered immobile only when it floated in the water without struggling. After each experiment, the used water was changed with fresh water.

Tail suspension test (TST)

The mouse was suspended by its tail in a box (25 cm long, 25 cm wide, and 30 cm high) with its head 5 cm above the bottom of the box. The test was also conducted in a relatively quiet environment as described before. The mouse was suspended for 6 min, and the immobility time

was recorded in the last 4 min. The mouse was considered immobile when it was passive and completely immobile. The test process was recorded with a monitoring system and analyzed by a researcher.

ELISA

After the depression-related behavioral tests, all mice were sacrificed. Serum was obtained from the abdominal artery, and brain tissue was also collected from each mouse and stored in a freezer at -80 °C for further analysis. SOD (Superoxide dismutase), MDA (Malondialdehyde), and GSH-Px (Glutathione peroxidase) were tested using ELISA kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). Expression levels of 5-hydroxytryptamine (5-HT), norepinephrine (NE), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) in the hippocampal tissue and prefrontal cortex tissue were all measured by ELISA kits (R&D system, USA) according to the protocol provided in the kit. The concentration was set according to the manual of the kit and was calculated with reference to the standard curve.

Western blot

Mice were sacrificed after the behavioral tests. Under disinfected conditions, the hippocampal tissue and prefrontal cortex tissue were quickly removed from each mouse brain and kept in cold (4 °C) saline solution. Tissue samples were homogenized in radioimmunoprecipitation assay lysis buffer (RIPA lysis buffer, containing protease inhibitor), then centrifuged at 12,000 ×g for 15 min. The supernatant was then collected at 4 °C. The BCA protein assay and western blot were performed to determine protein concentration. The proteins were separated by SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Millipore Company, Bedford, Mass.). The membrane was blocked with 5% bovine serum albumin at room temperature for 1 h, and the primary antibody was incubated overnight at 4 °C. Then, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. The main antibodies used included toll-like receptor type 4 (TLR4) (1:1,000), p-p38/p38 (1:1,000), p-NF- κ B/NF- κ B (1:1,000), NLRP3 (1:1,000), and caspase-1 (1:1,000). The membrane was then incubated with the enhanced chemiluminescence reagent (Perkinelmer) and secondary antibody (Chemicon). Data was obtained by a molecular imager.

Table 1 Effect of safflower extract on sucrose preference

Groups	0 week	2 weeks	4 weeks	6 weeks
Control	79.2±3.5	82.1±4.1	82.4±4.3	84.7±4.7
CUMS	82.1±4.1	71.5±3.2*	50.2±2.8*	39.6±2.4*
CUMS + SE, 10 mg/kg	81.0±5.2	70.8±4.5*	55.7±3.7 [#]	59.5±4.1* [#]
CUMS + SE, 30 mg/kg	81.4±4.8	69.7±4.2*	57.3±3.9 [#]	74.8±4.5* [#]
CUMS + Cli-095	79.8±5.1	70.6±4.6*	61.3±4.1 [#]	77.4±4.7* [#]
CUMS + Flu	80.5±4.7	71.2±4.1*	61.9±3.7 [#]	80.1±4.9* [#]

Compared with control, *P<0.05; compared with CUMS, [#]P<0.05. CUMS, chronic unpredictable mild stress; SE, safflower extract; Flu, fluoxetine hydrochloride.

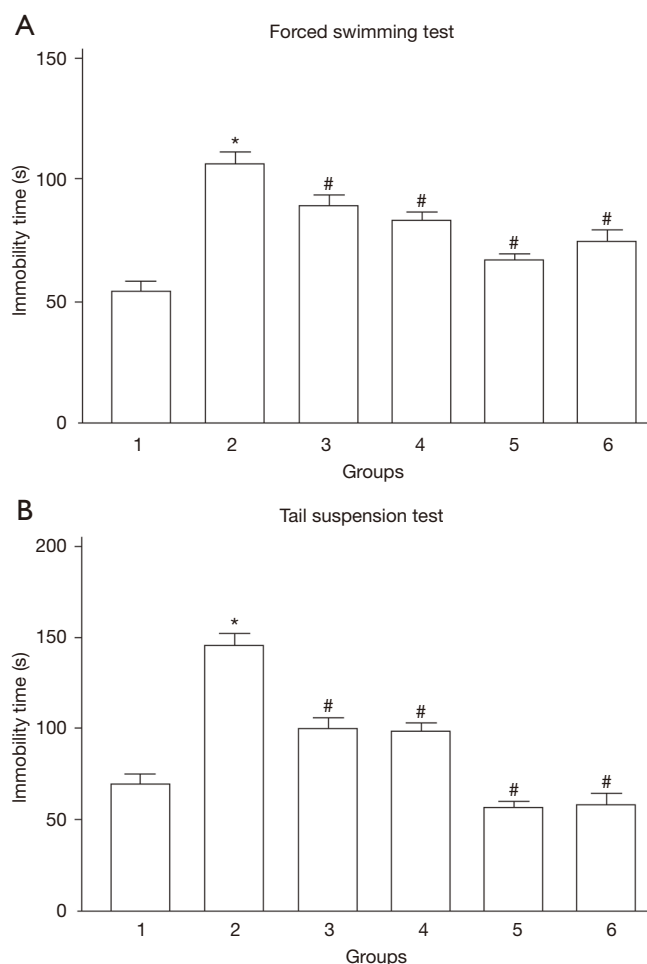


Figure 1 The effect of safflower extract on the depression-like behaviors of mice. (A) Forced swim test. (B) Tail suspension test. Groups: 1. Control; 2. CUMS; 3. CUMS + SE, 10 mg/kg; 4. CUMS + SE, 30 mg/kg; 5. CUMS + Cli-095; 6. CUMS + Flu. *, compared with control, P<0.05; [#], compared with CUMS, P<0.05. CUMS, chronic unpredictable mild stress; SE, safflower extract; Flu, fluoxetine hydrochloride.

Statistical analysis

GraphPad InStat 5.0 software was used for statistical analysis. Analysis of variance and Tukey's multiple comparison test were used. The results are expressed as mean ± standard deviation. Statistical significance was considered if P<0.05.

Results

The effect of SE on the SPT

After 6 weeks of observation and intervention, CUMS significantly reduced the SP of treated mice compared with the control group. Mice who received SE treatment (10 or 30 mg/kg) significantly consumed more SS compared with mice in the CUMS only group. After 4 weeks of treatment, mice treated with FLU (10 mg/kg) and Cli-095 (3 mg/kg) also showed increased SP compared with the CUMS only group. The increase in SP indicates that SE has a strong antidepressant effect in CUMS-exposed mice (Table 1).

The effect of SE on the depression-like behaviors of mice

The results of the OFT showed no significant difference in total distance between the groups. Compared with the control group, CUMS significantly increased the immobility time in the other 5 groups, which is consistent with the results of the SPT (Figure 1A,B). Treatment with SE (10 or 30 mg/kg) could alleviate the stress induced by CUMS and reduce the immobility time in the FST (Figure 1A) and TST (Figure 1B). FLU (10 mg/kg, for example) and Cli-095 (3 mg/kg, for example) treatments also significantly alleviated CUMS-induced stress and reduced the immobility time in the FST and TST (Figure 1).

Table 2 The effect of safflower extract on 5-HT and NE levels and inflammatory cytokines in mice ($\bar{X} \pm S$)

Tissue	Group	5-HT (pg/mg)	NE (pg/mg)	TNF- α (pg/mL)	IL-1 β (pg/mL)	IL-6 (pg/mL)
PC	CON	485.63 \pm 33.62	212.35 \pm 19.85	28.65 \pm 2.35	17.63 \pm 1.03	12.63 \pm 1.96
	CUMS	289.52 \pm 26.38	103.65 \pm 18.23	79.65 \pm 8.95	68.65 \pm 6.23	38.42 \pm 4.32
	CUMS + SE, 10 mg/kg	412.36 \pm 12.65	123.35 \pm 15.68	63.52 \pm 7.21	48.63 \pm 4.65	28.63 \pm 3.56
	CUMS + SE, 30 mg/kg	419.86 \pm 18.96	158.63 \pm 15.92	58.28 \pm 6.99	38.65 \pm 4.23	25.62 \pm 3.21
	CUMS + Cli-095	436.85 \pm 29.62	178.35 \pm 16.85	43.65 \pm 6.53	32.23 \pm 3.95	18.96 \pm 2.68
	CUMS + Flu	463.35 \pm 27.85	195.63 \pm 21.24	42.31 \pm 4.21	21.20 \pm 2.03	22.53 \pm 2.86
H	CON	426.35 \pm 15.63	163.52 \pm 17.63	18.73 \pm 2.65	18.85 \pm 1.04	37.65 \pm 2.35
	CUMS	219.36 \pm 5.20	86.35 \pm 16.20	58.74 \pm 5.62	38.20 \pm 4.26	89.62 \pm 5.68
	CUMS + SE, 10 mg/kg	285.42 \pm 13.25	97.63 \pm 17.38	37.28 \pm 4.36	28.63 \pm 2.56	67.56 \pm 4.32
	CUMS + SE, 30 mg/kg	348.85 \pm 16.24	122.82 \pm 16.35	31.23 \pm 4.03	24.82 \pm 3.01	49.53 \pm 4.66
	CUMS + Cli-095	371.52 \pm 20.31	124.65 \pm 17.92	26.98 \pm 3.82	21.65 \pm 2.86	46.98 \pm 3.95
	CUMS + Flu	387.29 \pm 22.36	148.37 \pm 15.28	21.36 \pm 2.01	18.92 \pm 2.34	38.62 \pm 2.38

CON, control; 5-HT, 5-hydroxytryptamine; NE, norepinephrine; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; CUMS, chronic unpredictable mild stress; SE, safflower extract; Flu, fluoxetine hydrochloride; PC, prefrontal cortex; H, hippocampus.

The effect of SE on 5-HT and NE levels and inflammatory cytokines in mice

Compared with the control group, the concentrations of 5-HT and NE in the prefrontal cortex tissue and hippocampal tissue in the CUMS group decreased significantly after 6 weeks of treatment, while the levels of 5-HT and NE in the prefrontal cortex and hippocampus increased significantly after treatment with SE (10 or 30 mg/kg). FLU and Cli-095 treatment also reversed the decrease in 5-HT and NE levels in the brain. In addition, serum 5-HT and NE levels in the CUMS group were lower than those in the control group. However, intervention with SE at a dose of 30 mg/kg significantly increased serum 5-HT and NE levels. TNF- α , IL-1 β , and IL-6 in the prefrontal cortex and hippocampus of CUMS mice were significantly increased, and SE treatment effectively reduced the increase in pro-inflammatory cytokines. This indicates that the anti-inflammatory effects of SE may contribute to its antidepressant effect (Table 2).

The effect of SE on the TLR4/p38/NF- κ B signaling pathway

Our results showed that CUMS significantly upregulated the expression levels of TLR4 and p-p38 in the prefrontal cortex and hippocampus (Figure 2A,B). Administration

of SE (10 or 30 mg/kg) or FLU (10 mg/kg) remarkably decreased the CUMS-induced increases in TLR4 and p-p38 (Figure 2A,B). SE could also significantly inhibit the CUMS-induced upregulation of p-NF- κ B in the prefrontal cortex and hippocampus (Figure 2C,D).

The effect of SE on the expression of the NLRP3 inflammasome

The NLRP3 inflammasome plays an important role in promoting the maturation of inflammatory cytokines. Evidence also shows that the NLRP3 inflammasome is involved in depression-like animal models. The potential effect of SE on the NLRP3 inflammasome in CUMS-treated mice was studied. CUMS induced the upregulation of NLRP3 and caspase-1 in the prefrontal cortex and hippocampus. Compared with the CUMS group, the decrease of NLRP3 and caspase-1 were obviously reversed after administration of SE (10 or 30 mg/kg) or FLU (10 mg/kg). In addition, administration of Cli-095 (3 mg/kg) could significantly inhibit the activation of NLRP3 and caspase-1 in the prefrontal cortex and hippocampus induced by CUMS, as shown in Figure 3.

Discussion

In the present study, we showed that depression-like

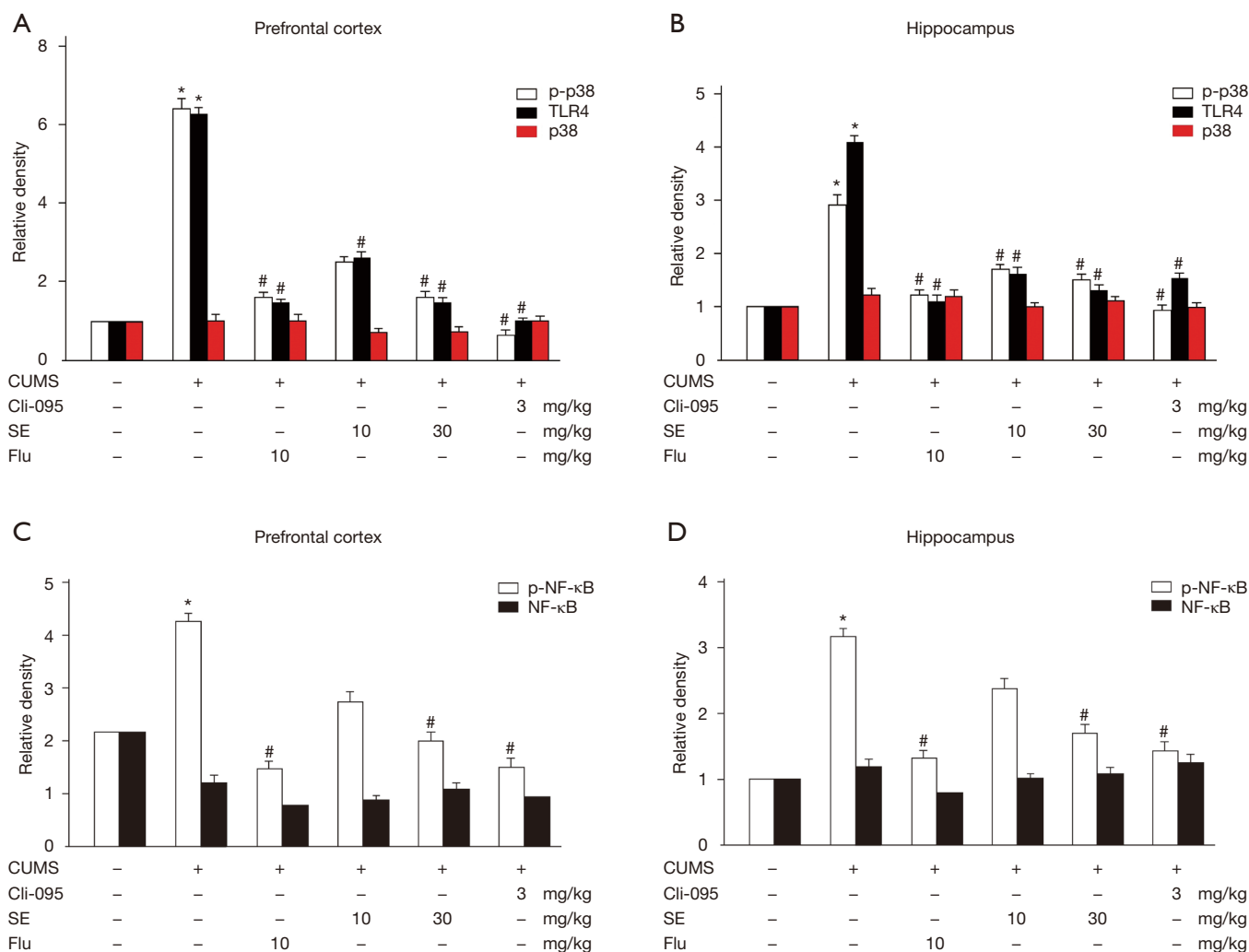


Figure 2 The effects of safflower extract on the TLR4/p38/NF- κ B signaling pathway (relative density derived from western blot image). Compared with control, * $P < 0.05$; compared with CUMS, # $P < 0.05$. CUMS, chronic unpredictable mild stress.

behaviors (such as impaired SP and prolonged immobility time induced by CUMS) could be improved by SE. In addition, SE decreased the CUMS-induced upregulation of 5-HT and NE expression in prefrontal cortex and hippocampal tissues. SE also reduced the increases in serum inflammatory cytokine levels (including TNF- α , IL-1 β , and IL-6), and inhibited the upregulation of TLR4/p38/NF- κ B/NLRP3 pathway-related proteins in prefrontal cortex and hippocampal tissues. Therefore, this study demonstrated that SE has a potential antidepressant effect on CUMS-induced depressive mice.

Previous related studies have shown that CUMS is widely used in establishing animal behavioral models of depression with satisfying effectiveness and reliability (8).

In our present study, we used CUMS to establish a mouse model of depression successfully. Using behavioral tests, mice treated with CUMS all showed depression-like behaviors. Compared with the controls, these mice drank less SS in the SPT and had longer immobility time in the FST and TST. According to previous studies, these manifestations suggested that these mice had depression-like symptoms. Furthermore, our experiments showed that treatment with SE for 4 weeks could significantly improve these depression-like behaviors through increasing SP and reducing the immobility time. Our study is the first, to the best of our knowledge, to provide new experimental evidence for the beneficial effect of SE in a CUMS-induced depression animal model. Excessive secretion of pro-

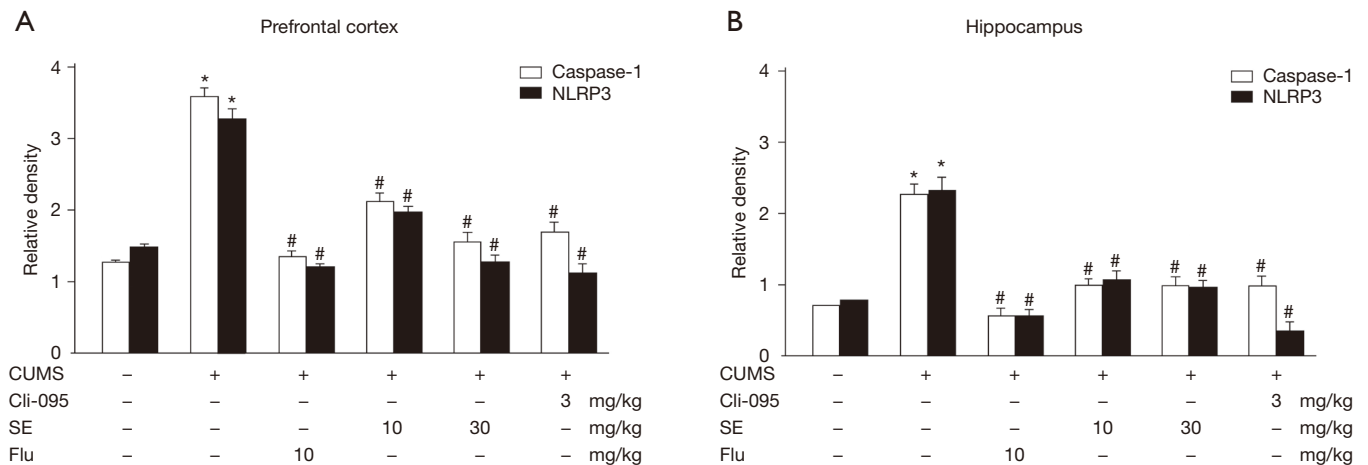


Figure 3 The effect of safflower extract on the expression of the NLRP3 inflammasome (relative density derived from western blot image). Compared with control, * $P < 0.05$; compared with CUMS, # $P < 0.05$. CUMS, chronic unpredictable mild stress.

inflammatory cytokines can lead to the downregulation of neurotrophic factors and inhibition of hippocampal neurogenesis, which can lead to memory and cognitive impairment (9). Inhibition of inflammation can improve the depressive behaviors of mice, which is consistent with our research results. The results showed that CUMS could significantly increase the expression levels of TNF- α , IL-1 β , and IL-6 in circulating blood and the prefrontal cortex and hippocampal tissues. In SE-treated mice, the increases in the expression levels of TNF- α , IL-1 β , and IL-6 were inhibited, suggesting that the beneficial effect of SE on depression may be attributed to its role in the inhibition of inflammation.

Some previous studies have demonstrated that TLR4 is significantly involved in the occurrence and progression of depression (10-12). Stress could significantly increase the expression levels of TLR4 and NF- κ B in the hippocampus, which could be alleviated by gene knockout of TLR4. In this study, we also found that SE could significantly prevent the increase of TLR4 and p-p38 in the prefrontal cortex and hippocampal tissue induced by CUMS. In addition, Cli-095 could also significantly inhibit the increase of TLR4 and p-p38 in the prefrontal cortex and hippocampal tissues, and improve the depressive behaviors induced by CUMS. NF- κ B is an important transcription factor which plays a key role in the regulation of immune responses. Neuroscientific studies have also shown that after activation, NF- κ B plays an important role in cognitive dysfunction, which is a manifestation of depression. In clinical studies, it has been found that somatic and social stress can both activate

the NF- κ B signaling pathway in depressive patients. In animal studies, investigators also found that the activation of NF- κ B signals induced by chronic stress contributes to depression-like behaviors and reduces the proliferation of neural stem cells in rodents (13). Since lipopolysaccharide (LPS) strongly stimulates the expression of IL-1 β and the activation of NF- κ B, these depression-related behaviors have also been found in animal models of acute depression induced by LPS (14). In addition, inhibition of NF- κ B signals could alleviate the negative effects of chronic stress on the SP of animals, which indicates that inhibiting NF- κ B signals may treat depression effectively (15). At the same time, our results showed that the effect of CUMS on the activation of the TLR4-p38-NF- κ B signaling pathway was significantly decreased by SE.

The NLRP3 inflammasome, as many studies have demonstrated, is an important downstream signaling molecule of TLR4 (16). Previous studies have shown that the NLRP3 inflammasome plays a key role in the process of inflammation (17). In molecular experiments and animal model experiments, the NLRP3 inflammasome can be easily activated by various stimuli, including different types of stressors and inflammatory cytokines (18). Recently, clinical and preclinical studies have revealed that the NLRP3 inflammasome can be activated in depressive patients and in animal models of depression (5). This study found that CUMS induced the activation of the NLRP3 inflammasome in the prefrontal cortex and hippocampal tissues, indicating that chronic stress-induced depression is related to the NLRP3 inflammasome-dependent

inflammatory response (19). Continuous treatment with SE for 4 weeks decreased the expression level of NLRP3 upregulated by CUMS. In addition, Cli-095 also inhibited the activation of the NLRP3 inflammasome induced by CUMS. In short, these findings demonstrated that SE could treat depression effectively, possibly through the inhibition of the TLR4-NF- κ B-NLRP3 signaling pathway.

In summary, SE has antidepressant effects in the CUMS-induced depression mouse model. The results of this study showed that SE can inhibit the TLR4-NF- κ B pathway induced by CUMS and regulate the activation of the NLRP3 inflammasome in CUMS mice. In addition, SE also showed effects similar to FLU, which provides a theoretical basis for SE in the treatment of depression. Based on these results, we assume that safflower extract can be treated to improve the effects of chronic mild stress on emotional learning and long-term potentiation in the hippocampal accumbens which will be verified in future study. This study has some limitations. First, this is an animal experiment which is different from the condition in human beings. Second, this study has no positive control. The protocol of this study was prepared before the study without registration.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/apm-21-1728>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal experiments were approved by the Animal Experiment Ethics Committee (AEEC) of Zhongshan School of

Medicine and were in compliance with the Guide for the Care and Use of Laboratory Animals.

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