

# Curcumin Ameliorates Chronic Mild Stress-Induced Depressive-Like Behavior via HMGB1/TLR4/NF- $\kappa$ B Signaling Pathway

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## Research

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# Abstract

## Background

Depression, one of the most frequently-occurring psychiatric disorders worldwide, is a significant inflammatory disorder. The polyphenol curcumin (Cur), which is extracted from *Curcuma longa*, has marked anti-inflammatory and anti-oxidative effects against inflammatory diseases. However, whether Cur has antidepressant effects and the possible mechanisms, are unclear. The present study aimed to assess Cur's beneficial effects on depressive-like behaviors using a chronic unpredictable mild stress (CUMS) model and its possible molecular mechanisms.

## Methods

We performed CUMS treated Sprague Dawley (SD) rats as a model of depression. Behavioral observations were performed by sucrose preference test (SPT), force swimming test (FST) and tail suspension test (TST). Hippocampal expression of oxidative stress markers and inflammatory cytokines were measured with ELISA. Hippocampal expression of high-mobility group box 1 (HMGB1), IL-1 $\beta$ , TNF- $\alpha$  and IL-6 were determined with quantitative PCR analyses and immunofluorescent staining. Hippocampal Toll-like receptor 4 (TLR4) and NF- $\kappa$ B activation were examined with Western blotting analysis.

## Results

Rats subjected to CUMS demonstrated marked depressive-like behavior (decreased locomotor activity and sucrose intake, and prolonged immobility). Their levels of oxidative stress and inflammatory cytokines increased significantly, and their levels of phosphorylated nuclear factor kappa-B (NF- $\kappa$ B), toll-like receptor 4 (TLR4), and HMGB1, also increased in the hippocampus. The changes were ameliorated significantly by treatment with Cur (50, 100 mg/kg) to varying degrees.

## Conclusion

This study demonstrated that Cur has a potent antidepressant effect via the HMGB1/TLR4/NF- $\kappa$ B pathway, suggesting that Cur might be a promising therapeutic drug for depression.

## Background

Among psychiatric disorders, depression is one of the most frequently-occurring, and is clinically characterized by feelings of guilt, cognitive dysfunction, body disturbances, and suicidal tendencies [1]. The World Health Organization (WHO) estimates that depression could become the second leading cause of disability after heart disease by 2030 [2, 3]. The pathophysiological symptoms of depression include deficiencies of inflammatory responses, cellular proliferation, dysregulated secretion of cytokines, and neuroplasticity [4]. Inflammation has been reported as an essential molecular mechanism of neuropsychiatric disorders, such as major depression [5, 6]. Depressive-like behavior might be closely related to elevation of inflammatory cytokines driven by oxygen stress, such as tumor necrosis factor- $\alpha$

(TNF- $\alpha$ ), Interleukin-6 (IL-6), and Interleukin-1 beta (IL-1 $\beta$ ) [7, 8], which are also closely associated with depressive symptoms [9, 10]. Therefore, decreasing proinflammatory cytokine levels is an important method to treat depression [8, 11, 12]. The identification of the optimal anti-inflammatory drugs for depression has become an urgent problem and requires further investigation.

In recent years, Chinese herbal extracts have been used as adjuvant therapy for neuroinflammatory diseases due to their anti-inflammatory activities [13–15]. For example, the protective effects of Baicalin, apigenin, and Tanshinone IIA (Tan IIA) have been reported [8, 16, 17]. Rhizome extracts of *Curcuma longa* Linnéa provide curcumin, a highly pleiotropic compound that interacts with inflammatory cytokines [18], which has been used as a remedy on to treat chronic inflammatory conditions, including cancers, chronic anterior uveitis, arthritis, pancreatitis, and inflammatory bowel disease [19–22]. In the treatment of these chronic inflammatory diseases, curcumin demonstrates marked anti-inflammatory and anti-oxidation effects. *In vitro* and *in vivo* studies showed that curcumin might act as a TNF blocker by binding directly to TNF [23, 24]. Further investigation showed that curcumin alleviates oxidative stress and inflammation during the modulation of lung carcinogenesis via nuclear factor erythroid-2 related factor 2 (NRF2)-kelch like ECH associated protein 1 (KEAP1) signaling [25]. Recent research demonstrated a certain therapeutic effect of curcumin on depression; however, the specific mechanism remains unclear.

The highly conserved non-histone nuclear protein, high-mobility group box-1 (HMGB1), is a member of the damage-associated molecular patterns (DAMPs) or alarmins group of proteins [26, 27]. When cells are necrotic or damaged, HMGB1 in the nucleus can be passively released, which induces pro-inflammatory cytokines secretion by macrophages [28]. Meanwhile, HMGB1 could be actively secreted by activated immune cells in response to inflammatory signals [29]. Excess HMGB1 in the extracellular space binds with many receptors on the cell surface, such as Toll-like receptor 4 (TLR4) [30], and plays a crucial part in chronic and acute inflammation pathogenesis (e.g., in autoimmune diseases, atherosclerosis, cancer, and sepsis) [26]. Increased HMGB1 levels in the central and peripheral nervous systems were observed in mice displaying symptoms of depression, leading to the proposal that that HMGB1 drives depression resulting from chronic stress [31, 32]. Moreover, inflammation mediated by HMGB1 in depressive-like behaviors might involve an imbalance between neurotoxic and neuroprotective factors, dysregulated kynurenine signaling, proinflammatory cytokines, and the Nod-like receptor family pyrin domain containing three (NLRP3) inflammasome [33].

Our previous experiment showed that Curcumin (Cur) reduced HMGB1 expression of in a rat model of chronic unpredictable mild stress (CUMS). Therefore, we hypothesized that Cur might ameliorate the induction of depressive-like behaviors by CUMS through the regulation of HMGB1 expression. Based on this hypothesis, we detected hippocampal TLR4/NF- $\kappa$ B pathway activation, inflammatory cytokines, markers of oxidative stress, and depressive-like behaviors to investigate the possible molecular mechanisms.

## Methods

## Animals

Sprague Dawley (SD) rats (male; approximately 6 to eight weeks old;  $200 \pm 20$  g) were obtained from the Experimental Animal Center of Air Force Military Medical University (Xi'an, China). The animals were reared under conditions of  $25 \pm 2^\circ\text{C}$ , light/dark cycle = 12 hours, and *ad libitum* access to water and food. The animal experiments were carried out according to The National Institutes of Health guidelines for care and use of Laboratory Animals and were approved by the relevant ethics committee.

## Grouping and treatment

Rats ( $n = 60$ ) were assigned randomly to six groups: Control group, CUMS group, CUMS + Cur (50 mg/kg) group, CUMS + Cur (100 mg/kg) group, CUMS + Fluoxetine (Flu, antidepressant drug) (20 mg/kg) group, and CUMS + Glycyrrhizic Acid (Gly, HMGB1 inhibitor) (10 mg/kg) group, with ten rats in each group. Rats in the control group were maintained under normal conditions. Rats in CUMS groups were subjected to CUMS according to the protocol of a previous study [34–36], and were scheduled randomly and changed weekly (Table 1). Cur was dissolved in cocoa buffer at different concentrations; the other drugs were dissolved in water. All drugs were administered once per day between 08:00 and 09:00 h. The experimental procedure is shown in Fig. 1A. After a 7-day adaptation period, all rats, except the control group, were exposed to CUMS for 28 days. Drugs were administered to the specific groups and behavior tests were assessed 2 weeks after drug administration (between 09:00 and 12:00 h).

Table 1  
Chronic unpredictable mild stress  
(CUMS)-related stressors

<b>Stressor</b>	<b>Duration</b>
Restraint stress	8 hours
Noise	4 hours
Cold water-immersion	5 mins
Tail clamp	10 mins
Light/dark cycle reversal	12 hours
The cage is tilted	12 hours
The cage is soiled	12 hours

## Sucrose preference test (SPT)

A previously described protocol was used to carry out the SPT [37]. Before the test, all rats were acclimated to a 1%, w/v sucrose solution for 24 h as a training period. After 23 h of deprivation of food and water, the rats were fed with water and 1% sucrose solution at the same time. The consumption of

water and sucrose solution in the following 1 h were recorded. According to the ratio of sucrose solution consumption to total liquid, the sugar solution preference was obtained.

### Forced swimming test (FST)

A previously described protocol was used to carry out the FST, with a minor change [38]. In brief, individually, the rats were forced to swim in a glass cylinder (50 cm height · 20 cm diameter) containing water (30 cm height, 24 ± 2°C) for ten mins. A trained technician, who was blinded to the groups, recorded the rat's immobility time during the last 4 mins, which was defined as the time that rats relaxed with no movement except keep their head above water.

### Tail suspension test (TST)

A previously described protocol was used to carry out the TST, with a minor change [5]. Rats were suspended individually via their tails using adhesive tape (approximately 50 cm above the floor). The test lasted for 6 mins. The immobility time was recorded during the last 4 mins by a trained technician who was blinded to groups, which was defined as the time that rat relaxed without any struggling movement.

### RNA extraction and qRT-PCR

Total RNA extraction from the hippocampus was performed using the Trizol reagent (Bioteke, Beijing, China) according to the manufacturer's instructions. Complementary DNA was reverse transcribed using a PrimeScript RT Reagent Kit (Bioteke). Quantitative real-time PCR was performed using a 2 × Power Taq PCR MasterMix (Bioteke) with *Actb* (β-actin) as the internal control. All primers used in PCR were provided by Sangon Biotech (Shanghai, China) Co., Ltd and listed below:

*Hmgb1* (forward: TGA AGA TAT GGC AAA GGC; reverse: GGC GGT ACT CAG AAC AGA); *Il1b* (forward: TTC AAA TCT CAC AGC AGC AT; reverse: CAC GGG CAA GAC ATA GGT AG); *Il6* (forward: AAC TCC ATC TGC CCT TCA; reverse: CTG TTG TGG GTG GTA TCC TC); *Tnfa* (forward: CGG AAA GCA TGA TCC GAG AT; reverse: AGA CAG AAG AGC GTG GTG GC); *Actb* (forward: ACG TTG ACA TCC GTA AAG AC; reverse: TAG GAG CCA GGG CAG TAA).

### Determination of oxidative stress markers and inflammatory cytokines in the hippocampus

To measure the oxidative stress markers and inflammatory cytokines, rat hippocampi were excised, homogenized in 1.0% KCl, centrifuged at 4°C for 10 min, and the supernatant retained. Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the levels of pyruvate dehydrogenase (PDH), superoxide dismutase (SOD), glutathione (GSH), reactive oxygen species (ROS), IL-1β, IL-6, and TNF-α, following the manufacturer's protocols.

### Immunofluorescence staining

After the behavioral tests, intracardial perfusion was used to sacrifice the rats. The hippocampi were excised and a cryostat (CM1800, Leica, Wetzlar, Germany) was used to prepare 20 μm longitudinal

sections. Then, according to a previously described method, immunofluorescence staining was carried out [38]. The sections were visualized under a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan). The following primary antibodies were used: rabbit anti-IL-1 $\beta$  (1:100, Bioworld Technology, Bloomington, MN, USA), rabbit anti-HMGB1 (1:100, Bioworld), and rabbit anti-TNF- $\alpha$  (1:200, Bioworld).

### Western blotting

Samples of the hippocampus were cut into small pieces and homogenized in ice-cold Radioimmunoprecipitation assay (RIPA) buffer. The samples were centrifuged and a bicinchoninic acid (BCA) kit (Wanlei Biotechnology, Xi'an, China) was used to determine the protein concentration in the supernatant. The protein samples were subjected to 12% Tris-glycine SDS-PAGE, electrotransferred to polyvinylidene fluoride membranes, blocked, and incubated with antibodies against HMGB1 (1:500, Bioworld), TLR4 (1:500, Bioworld), NF- $\kappa$ B (1:500, Bioworld), phosphorylated (p)-NF- $\kappa$ B (1:500, Biorbyt, Cambridge, UK), and  $\beta$ -actin (1:5000, Bioworld) at 4°C overnight. The membranes were then incubated for 2 h at room temperature with secondary antibodies (1:5000, Bioworld). A ChemiScope 6200 (Clinx Science Instruments, Shanghai, China) captured the signal from the immunoreactive proteins, and the band levels were quantified using the Clinx Image Analysis software.

## Statistical analysis

All data are shown as the mean  $\pm$  the standard error of the mean (SEM). The data were analyzed using one way analysis of variance (ANOVA) together with Student-Newman-Keuls (SNK-q) test in SPSS 22.0 (IBM Corp., Armonk, NY, USA). Statistical significance was indicated by  $P < 0.05$ .

## Results

### Cur alleviated depressive-like behavior in CUMSs exposed rats

#### Cur effects on sucrose consumption

Compared with that in the control group, the sucrose consumption in the CUMS group decreased significantly. Administration of Cur (50, 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) increased the sucrose consumption significantly compared with that in the CUMS group (Fig. 1B). No significant difference was detected among the control, Cur (50, 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) groups.

#### Effects of Cur on the immobility time in FST and TST

As shown in Fig. 1C and Fig. 1D, compared with the control group, a significant increase in the immobility time was observed in the CUMS group in the FST and TST tests. Administration of Cur (50, 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) significantly decreased the immobility time compared with that in the CUMS group. The immobility times were not significantly different among the control, Cur (50, 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) groups.

## **Cur inhibited the level of oxidative stress in hippocampus exposed to CUMS**

As shown in Fig. 2, ROS expression increased significantly in the CUMS group compared with that in the control group. Meanwhile, GSH, SOD, and PDH activities were reduced significantly in the CUMS group compared with those in the control group. By contrast, treatment with Cur (50, 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) decreased the hippocampal ROS generation and increased the activities of GSH, SOD, and PDH to varying degrees compared with those in the CUMS group. These activities and ROS levels were not significant different among the control, Cur (50, 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) groups.

## **Cur reduced the inflammatory cytokine levels in the hippocampus exposed to CUMS**

As shown in Fig. 3A-3F, inflammatory cytokine levels, including TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , increased significantly in the CUMS group compared with those in the control group. Cur (100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) treatment resulted in decreased inflammatory cytokine levels in the hippocampus to varying degrees compared with those in the CUMS group. There was no significant difference among the control, Cur (100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) groups. There was no significant difference between the Cur (50 mg/kg) and the CUMS groups.

As shown in Fig. 3G, HMGB1, IL-6, and IL-1 $\beta$  levels increased in the hippocampal cytoplasm of the CUMS group compared with those in the control group. Contrastingly, the Cur (50 and 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) treatments resulted in varying degrees of reduction in the levels of these cytokines in the hippocampus compared with those in the CUMS group. Their levels among the Cur (100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) groups were not significantly different.

## **Cur regulated oxidative stress and inflammatory cytokines via NF $\kappa$ B/ HMGB1/ TLR4 signaling**

As shown in Fig. 4, compared with those in the control group, the CUMS group had increased levels of p-NF- $\kappa$ B p65, TLR4, and HMGB1, while the treatment with Cur (50 and 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) dramatically restored their levels, to varying degrees. Among the control, Cur (50 and 100 mg/kg), Flu (20 mg/kg), and Gly (10 mg/kg) groups, there were no significant differences.

## **Discussion**

Depressive disorder has a prevalence of 4.4% worldwide [39]. Therefore, the discovery of new therapeutic targets to treat depressive disorder is imperative. Recent research suggests that the pathogenesis of depression involves neuroinflammation and oxidative stress injury [17], which is an important direction of

anti-depressive therapy [8, 11, 12]. Curcumin has powerful anti-inflammatory and antioxidation effects in chronic inflammatory disease; however, Cur's effects on depression and its mechanism are unclear. The results of the present study showed that CUMS-induced depressive-like behavior in rats could be ameliorated by treatment with Cur. Furthermore, Cur treatment exhibited antidepressant effects by ameliorating oxidative stress and neuroinflammation in the hippocampus, and the underlying mechanisms might be associated the HMGB1/TLR4/NF- $\kappa$ B pathway.

Patients with depression generally display many behavioral abnormalities, such as low mood, slow thinking, and cognitive function damage. Thus, the potential antidepressive activity of a drug are frequently assessed using behavioral experiments. The CUMS model is a recognized animal model of stress depression [34–36]. It is reported that rats exposed to CUMS displayed phenotypes resembling depression and showed long-term changes to motivational states, anhedonia, and behavioral coping, which could be improved by treatment with antidepressants [40]. Our results showed that the rats in the CUMS group showed significantly decreased sugar preference and increased immobility time in the FST and TST tests. Cur administration displayed antidepressive effects in CUMS-induced rats, as did treatment with Flu and Gly.

The vital roles of inflammatory cytokines and oxidative stress in chronic stress-induced psychiatric disorders are well documented [8, 41]. In the pathogenesis of a depressive disorder, upon chronic stress, the balance between antioxidant and oxidant factors shifts, resulting in the production of excess inflammatory cytokines and ROS [42]. Many antidepressants act via their anti-inflammatory and anti-oxidative effects [43]. Cur, a natural antioxidant, can capture or scavenge free radicals directly because of the phenolic hydroxyl group in its structure [44]. Cur can exert a neuroprotective effect by inhibiting oxidative stress through the NRF2/protein kinase B (AKT) pathway, thereby preventing brain injury [45]. In TNF- $\alpha$ -treated HaCaT cells, Cur was observed to inhibit IL-1 $\beta$  and IL-6 expression via the NF- $\kappa$ B and MAPK pathways [46]. Herein, we revealed that Cur treatment increased antioxidant enzyme activities (PDH, SOD, and GSH), and decreased inflammatory factor expression (IL-6, TNF- $\alpha$  and IL-1 $\beta$ ). These results agree with those of previous studies that demonstrated curcumin's anti-depressive effects acting via its anti-inflammatory and anti-oxidative activities in CUMS.

The highly conserved nuclear protein HMGB1 is distributed widely in mammalian cells [47]. HMGB1 has become a hotspot of inflammation research because of its pro-inflammatory effect, acting as a strong mediator of inflammation linking infection and tissue damage [33]. In CUMS mice, the hippocampus [48] cerebral cortex [32] expressed high levels of HMGB1. Depression-like behavior can be induced by stress via NF $\kappa$ B/TLR4/HMGB1 signaling in the hippocampus [48]. In animal models, a reduced sucrose preference and prolonged immobility in TST and FST indicate that HMGB1 mediation of inflammation induces depressive-like behaviors, which is consistent with the results of our previous study. In addition, western blotting analysis revealed that treatment with Cur reversed the high hippocampal HMGB1 levels and those of its downstream molecules, p-NF- $\kappa$ B p65, and TLR4, in CUMS rats similarly to Gly treatment. Thus, we concluded that HMGB1/TLR4/NF- $\kappa$ B signaling mediates the protection provided by Cur on the inflammatory cytokines, oxidative stress, and depressive-like behavior in rats exposed to CUMS.

## Conclusion

This work provides evidence that Cur has anti-depressant activity in a chronic mild stress model. The antidepressant properties mostly derived from Cur's effects on inflammatory cytokine levels and oxidative stress related to NFκB/TLR4/HMGB1 signaling.

## Abbreviations

Cur	Curcumin
CUMS	Chronic unpredictable mild stress
Flu	Fluoxetine
SPT	Sucrose preference test
Gly	Glycyrrhizic Acid
TST	Tail suspension tests
FST	Forced swimming tests
DAMP	Danger-associated molecular pattern
HMGB1	High mobility group box 1 protein
ELISA	Enzyme-linked immunosorbent assay
DMSO	Dimethyl sulfoxide
TBST	Tris-buffered saline-tween

## Declarations

### Ethics approval and consent to participate

All animal experiments were approved by The Laboratory Animal Care & Welfare Committee, School of Stomatology, Fourth Military Medical University (NO. 2019-091).

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analysed during this study are included in this published article.

### Conflicts of Interest

The authors declare that they have no competing interests.

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## Authors' Contributions

LM conducted the experiments. LM, FH and WJ collected and analyzed the data. JW and YYS gave technical or material support. YJC and MZ contributed to the study design. LM, YYS and FH drafted the manuscript. MZ supervised the project. YJC and MZ obtained funding. All authors read and approved the final manuscript.

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No.

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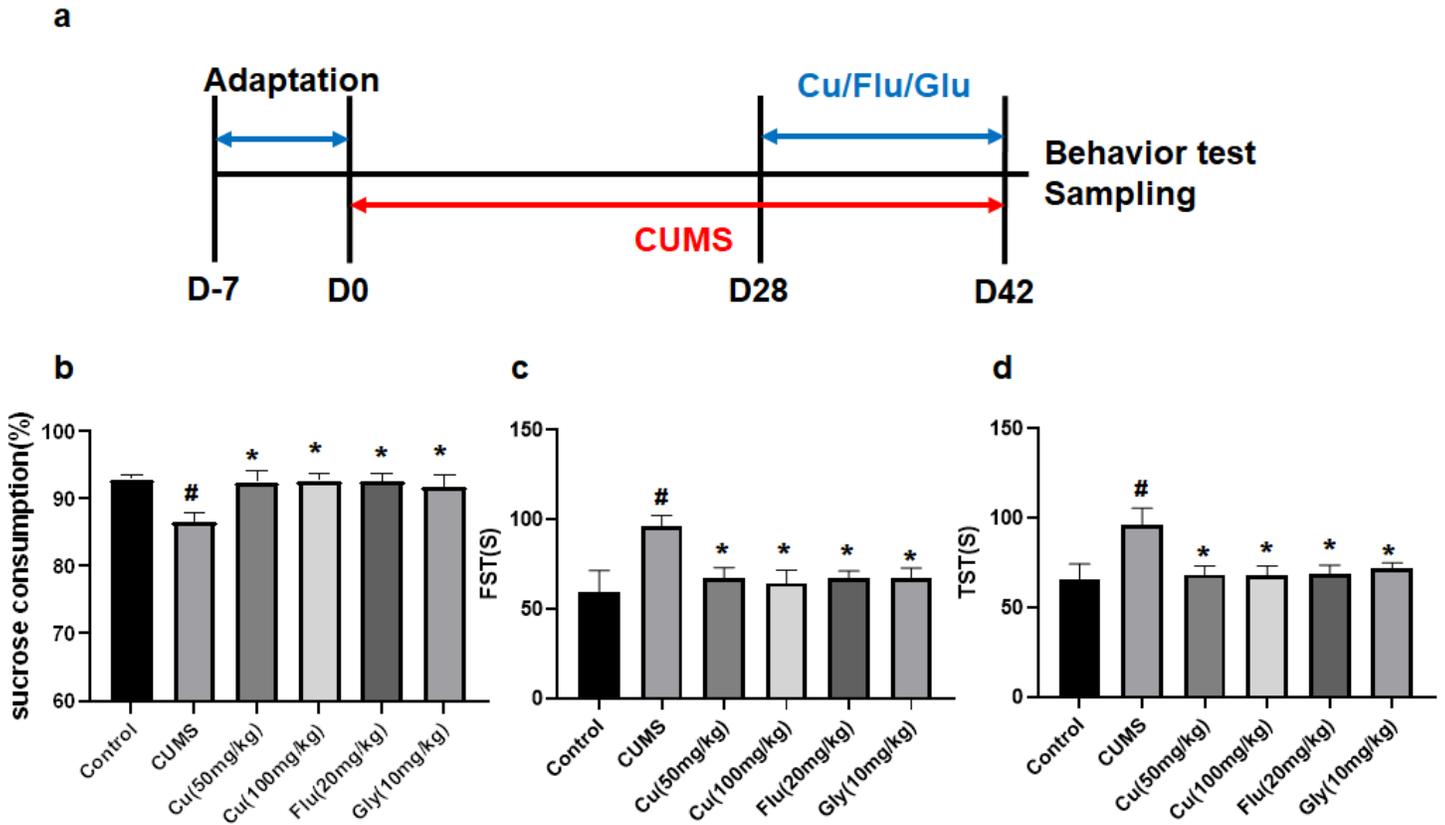
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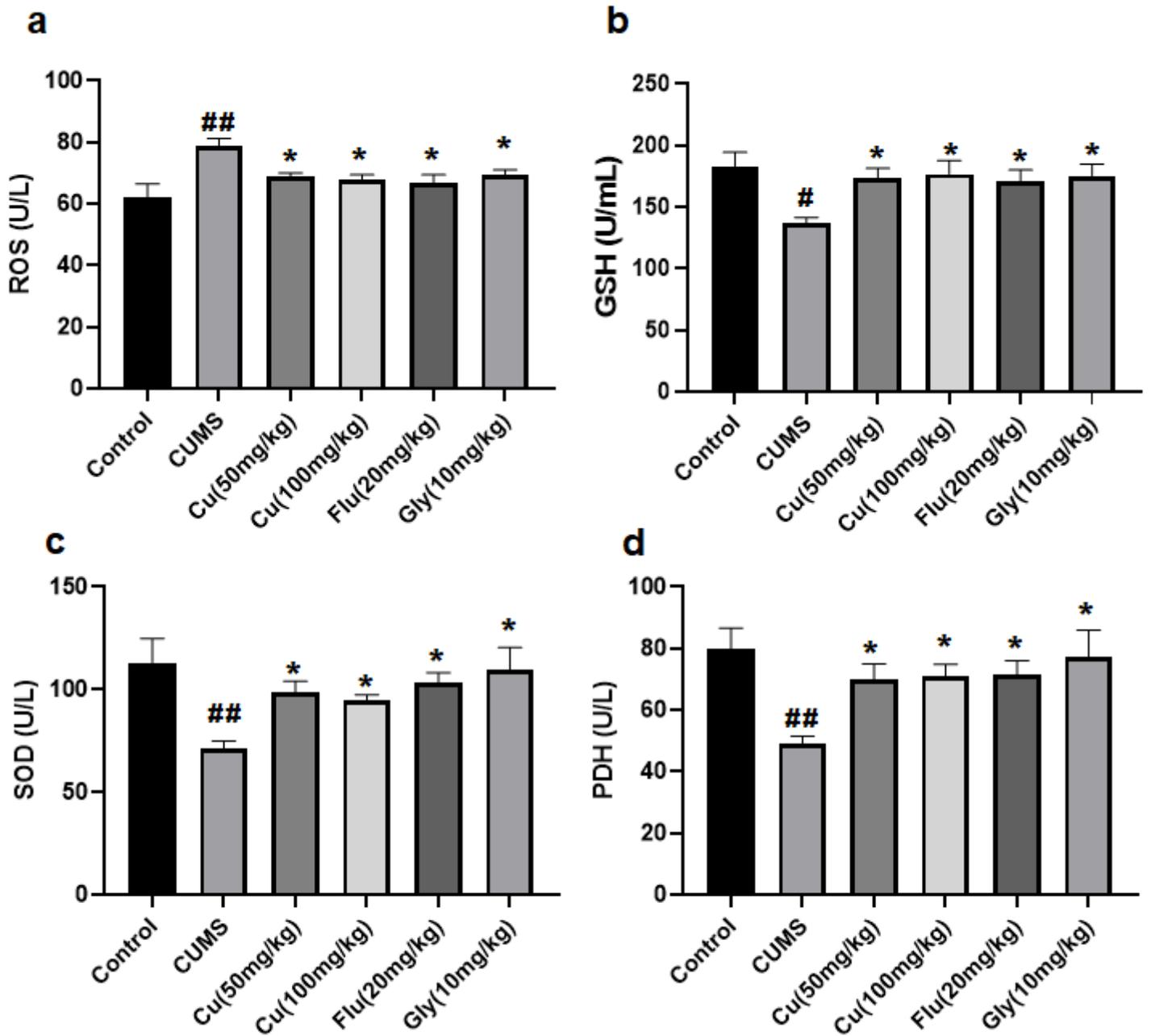
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## Figures



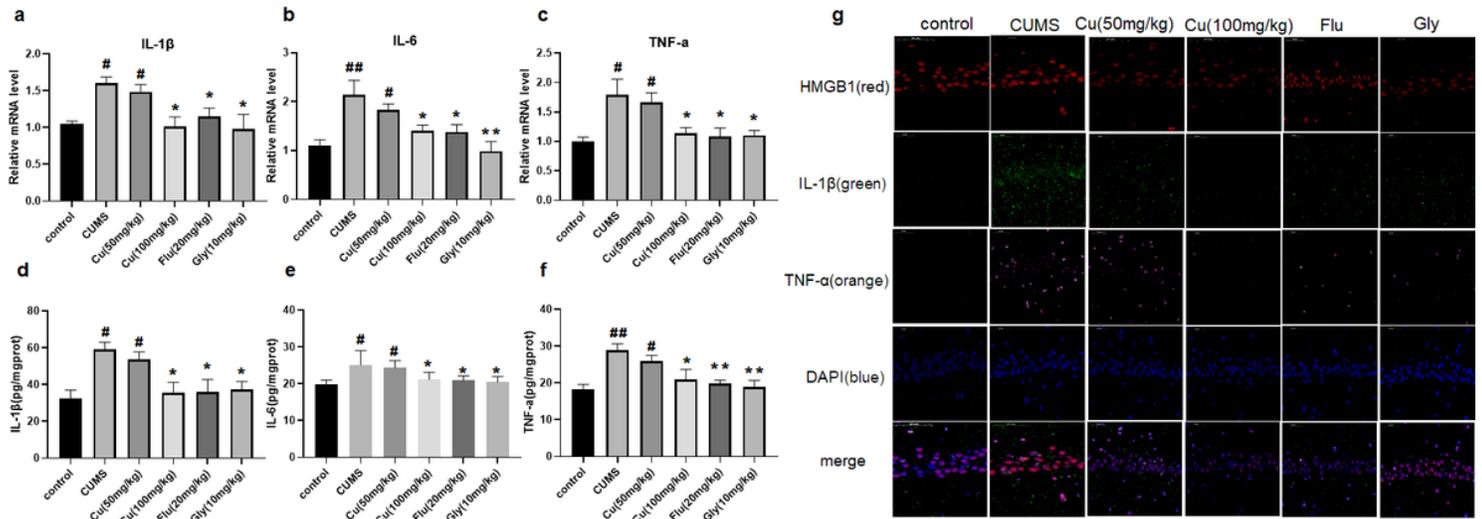
**Figure 1**

Curcumin ameliorated depressive-like behaviors in a rat model of CUMS. a. The experimental paradigm. b. Sucrose consumption in SPT. c. Immobility time in FST. d. Immobility time in FST. All data are expressed as the mean  $\pm$  SEM. # refers to  $p < 0.05$ , ## refers to  $p < 0.01$ , compared with the control group. \* refers to  $p < 0.05$ , compared with the CUMS group. Cur, curcumin; CUMS, chronic unpredictable mild stress; SPT, sucrose preference test; FST, forced swimming test; SEM standard error of the mean.



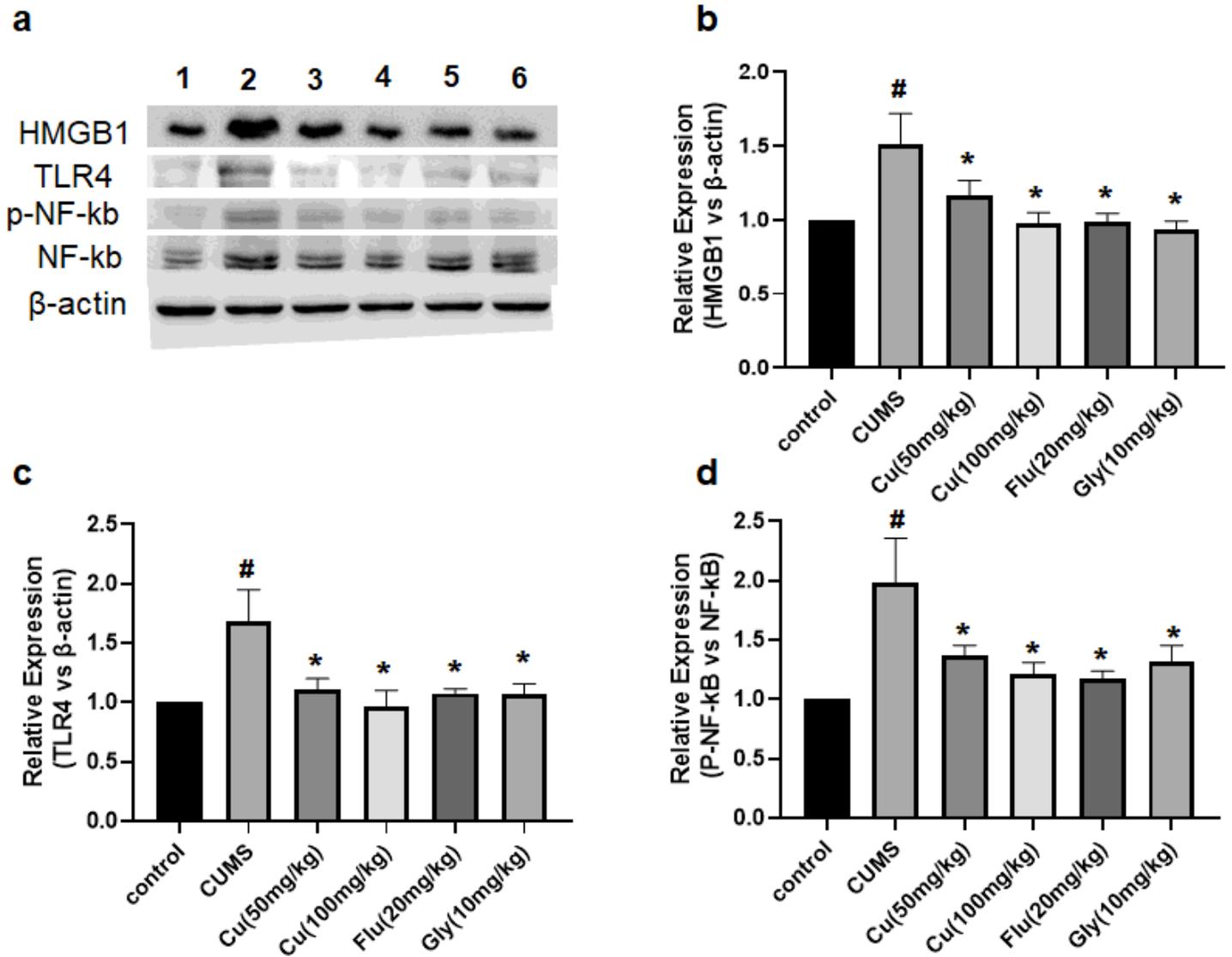
**Figure 2**

Curcumin inhibited the level of oxidation-related stress in CUMS-exposed hippocampi. a. ROS expression. b. Activity of GSH. c. Activity of SOD. d. Activity of PDH. Data are shown as the mean  $\pm$  SEM. # refers to  $p < 0.05$ , compared with the control group. ## refers to  $p < 0.01$ , compared with the control group. \* refers to  $p < 0.05$ , compared with the CUMS group. Cur, curcumin; CUMS, chronic unpredictable mild stress; GSH, glutathione; SOD, superoxide dismutase; PDH, pyruvate dehydrogenase; SEM standard error of the mean.



**Figure 3**

Curcumin inhibited the expression of inflammation-related cytokines in CUMS-exposed hippocampi. a and d. Expression of IL-1 $\beta$ . b and e. Expression of IL-6. c and f. Expression of TNF- $\alpha$ . g. Immunofluorescence staining of HMGB1, IL-1 $\beta$ , and TNF- $\alpha$  in hippocampus. Data are shown as the mean  $\pm$  SEM. # refers to  $p < 0.05$ , ## refers to  $p < 0.01$ , compared with the control group. \* refers to  $p < 0.05$ , \*\* refers to  $p < 0.01$ , compared with the CUMS group. Cur, curcumin; CUMS, chronic unpredictable mild stress; IL-1 $\beta$ , interleukin 1 beta; IL-6, interleukin 6; HMGB1, High mobility group box 1 protein; TNF  $\alpha$ , tumor necrosis factor alpha; SEM standard error of the mean.



**Figure 4**

Curcumin regulation of oxidative stress and inflammatory cytokines involved HMGB1/TLR4/NF κB signaling. a. Representative immunoblots for TLR4, NF-kB, p-NF-kB, HMGB1, and β-actin. 1: Control group, 2: CUMS group, 3: CUMS+ Cur (50 mg/kg) group, 4: CUMS+ Cur (100 mg/kg) group, 5: CUMS +Flu (20 mg/kg) group, 6: CUMS+ Gly (10 mg/kg) group. b. The relative expression level of HMGB1. c. The TLR4 relative expression level. d. The p-NF-kB relative expression level. Data are shown as the mean ± SEM. # refers to  $p < 0.05$ , compared with the control group. \* refers to  $p < 0.05$ , compared with the CUMS group. Cur, curcumin; CUMS, chronic unpredictable mild stress; HMGB1, High mobility group box 1 protein; TLR4, toll-like receptor 4; NF-κB, nuclear factor kappa B; SEM standard error of the mean.