

Protective Effects of Silibinin and Its Possible Mechanism of Action in Mice Exposed to Chronic Unpredictable Mild Stress

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Abstract

Silibinin, a natural flavonoid antioxidant isolated from extracts of the milk thistle herb, has recently been identified as having anti-hepatotoxic and anticancer properties. In this paper, we investigated the effects of silibinin on behavior and neuroplasticity in mice subjected to chronic unpredictable mild stress (CUMS). After 5 consecutive weeks of CUMS, the mice were treated with silibinin (100 mg/kg, 200 mg/kg and 400 mg/kg by oral gavage) for 3 consecutive weeks. The results showed that silibinin administration significantly alleviated the CUMS-induced depressive-like behavior, including the total number of squares crossed and the frequency of rearing in the open field test, the immobility time in the tail suspension test and the forced swimming test. Furthermore, silibinin treatment increased the levels of brain-derived neurotrophic factor (BDNF), serotonin (5-HT) and norepinephrine (NE) in the prefrontal cortex and hippocampus. Our study provides new insight into the protective effects of silibinin on the depressive status of CUMS mice, specifically by improving neuroplasticity and neurotransmission.

Key Words: Chronic unpredictable mild stress, Silibinin, Depression, BDNF, 5-HT, NE

INTRODUCTION

Depression is a mental disorder that is recognized as a complex syndrome of affective, vegetative and cognitive symptoms with considerable morbidity and mortality (Stewart *et al.*, 2003). However, the exact mechanism resulting in the development of depression is still not clear, and adverse effects played a significant role in the evolution of available, chemically synthesized antidepressants (George and O'Malley, 2004). Currently, plant-derived natural products and derivatives have received more attention regarding their use as therapeutic agents and of structural diversity (Li *et al.*, 2012).

Silibinin, the major pharmacologically active compound of silymarin, is well known for its hepatoprotective activities and cancer chemopreventive efficacy, which are explained by its potent anti-inflammatory and antioxidative effects (Souza *et al.*, 2012; Agarwal *et al.*, 2013). It has been reported that silibinin could protect the central nervous system from ethanol-induced brain injury (La Grange *et al.*, 1999), lipopolysaccharide (LPS)-induced neurotoxicity (Wang *et al.*, 2002), and

A β -induced recognition memory impairment (Lu *et al.*, 2012). However, it remains unclear whether silibinin has protective effects in the treatment of depression in mice.

Unpredictable chronic mild stress (CUMS), a well-validated animal model of depression, has been widely used to evaluate the antidepressant effects of diverse drugs (Liu *et al.*, 2014). As this model closely mirrors the clinical antidepressant treatment, we therefore decided to investigate the effects of silibinin treatment on reversing the behavioral and biochemical alterations in CUMS mice.

Brain-derived neurotrophic factor (BDNF) functions in both the peripheral and central nervous systems (CNS) and has a beneficial impact on supporting the survival of existing neurons and promoting the growth of newly differentiated neurons and synapses (Acheson *et al.*, 1995; Bergami and Berninger, 2012). Furthermore, BDNF itself is essential for long-term memory (Bekinschtein *et al.*, 2008). Several studies have revealed that altered BDNF gene expression is associated with the development of depression. Rats exposed to stress were reported to exhibit a 12.5-25% decrease in BDNF mRNA levels in the hippocampus (Warner-Schmidt and Duman, 2006).

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Additionally, clinical data have indicated that antidepressant administration can attenuate the serum BDNF abnormalities in depressed patients (Dwivedi *et al.*, 2003; Shimizu *et al.*, 2003). These data suggest a pathophysiological link between BDNF and depression progression.

The monoamine theory complements the neurotrophic hypothesis of major depressive disorder (Maletic *et al.*, 2007), and postulates that low levels of monoamines, particularly 5-HT and NE, are involved in the incidence of depression (Blier and El Mansari, 2013; Huang *et al.*, 2014). 5-HT is primarily found in platelets, the gastrointestinal tract and CNS, which is commonly thought to be a contributor to feelings of well-being and happiness (Young, 2007). NE is mainly responsible for cognitive alertness. When activated, NE neurons can affect large areas of the brain to promote senses of arousal and alertness and influence the reward system (Koob and Le Moal, 2001).

In the present study, we investigated whether silibinin administration in mice impacted the changes in behavior, neuroplasticity and neurotransmission in response to CUMS.

MATERIALS AND METHODS

Animals

Kunming mice (weighing 18-22 g, 6-8 weeks old, either sex) were purchased from the Experimental Animal Center of Xuzhou Medical College and were housed in a temperature controlled room ($22 \pm 2^\circ\text{C}$) on a 12/12-hr day/night cycle. The animals were randomized into specified experimental groups and allowed to acclimatize for 1 week before the experiment. Food and water were provided *ad libitum*. All experiments were performed between 7:30 p.m. and 10:30 p.m. and each animal was used only once. The procedures in this study were carried out under the approval of the Animal Ethics Committee of Xuzhou Medical College and complied with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No.85-23, revised 1985).

Experimental design

In this study, mice were separated into five groups ($n=12$ each): control+vehicle, CUMS+vehicle, CUMS+silibinin (100 mg/kg), CUMS+silibinin (200 mg/kg), CUMS+silibinin (400 mg/kg), and CUMS+fluoxetine (20 mg/kg). Doses were based on body weight. Silibinin (Sigma-Aldrich, USA.) was suspended in a 0.3% carboxymethylcellulose (CMC) solution and provided by oral gavage. Mice were subjected to various mild stressors for 5 weeks and then administered silibinin for 3 weeks.

CUMS procedures

The CUMS procedures were performed as previously reported (Gronli *et al.*, 2006; Yang *et al.*, 2009) with slight modifications. Mice in the experimental groups were exposed to stressors once daily for 5 consecutive weeks, including nip trail and cage tilting for 24 h, food deprivation for 48 h, water deprivation for 24 h, cold swimming (at 4°C) for 5 min, tail pinch (1 cm from the tip of the tail) for 1 min, damp bedding (200ml of water per cage) for 6 h and low intensity stroboscopic illumination in darkness for 8 h. Mice received one of these stresses per day, and the same stressor was not applied in 2 consecutive days.

Open field test

An open field (l, w, h (cm): $50 \times 50 \times 35$) with a 16-square grid floor was used (Kajiyama *et al.*, 2010). Motor activity was evaluated in wooden cages for 20 min. Mice were put into one of four test cages for 15 min to adapt to their surroundings. Two reliable measures of open field behaviors were scored. First, the total number of squares crossed were measured as the number of squares that each mouse crossed by placing at least the two front paws into a new square during 5 min. Second, rearing was measured as the number of times that each mouse had both front paws off the floor at the same time during 5 min, except when grooming. The open field was situated in an independent room. The box was thoroughly cleaned after each trial.

Tail suspension test

Mice were suspended by the tail for 5 min by attaching them to a horizontal bar (distance from the floor is 70 cm) with adhesive tape placed 2 cm from the tip of the tail, and positioned with their stomachs towards the investigator to assure the observation of total immobility (Steru *et al.*, 1985). Additionally, the mouse was 15 cm away from the nearest object and was both acoustically and visually isolated. Mice were considered immobile only when they hung passively and completely motionless; mice that climbed their tails during the trials were excluded from data analysis. This procedure was conducted by the same observer, who was blind to the specific experimental groups and strictly followed the test standard procedures.

Forced swimming test

The open field test was performed as previously described (Noda *et al.*, 1995). Mice were dropped individually into glass cylinders (h, d (cm): 19×14) containing 15 cm-deep water that was maintained at $23 \pm 1^\circ\text{C}$, and remained there for 6 min. A mouse was considered immobile when it floated in an upright position and made only very slight movements that were necessary to keep its head above water. Twelve mice in each group were tested simultaneously and the time of immobility was recorded during the last 4 min of the 6-min testing period, following 2 min of habituation. The test was performed by the same well-trained observer, who was blinded to the group assignment.

ELISA

Ten mice were killed from each group, and two different brain regions, the prefrontal cortex and hippocampus, were dissected for ELISA analysis. Brain tissues were homogenized in RIPA lysis buffer at 4°C . The homogenates were diluted and centrifuged at 3000 g for 20 min at 4°C , and the supernatant fractions were used for ELISA analysis.

Mouse BDNF ELISA kits (Shanghai Westang, China) were used to detect BDNF protein levels. The procedure was performed according to the manufacturer's instructions. Samples or standards (100 μl) were added to each well of 96-well plates that were coated with an anti-mouse BDNF antibody. The plates were incubated for 90 min at 37°C and then washed five times. A biotinylated specific antibody (100 μl) was added into each well and incubated for 60 min at 37°C . The plates were then washed, treated with 100 μl of diluted streptavidin-HRP and incubated for 30 min at 37°C . The color was produced by adding 100 μl of the substrate solution for 15 min after washing. Stop solution (100 μl) was added to terminate

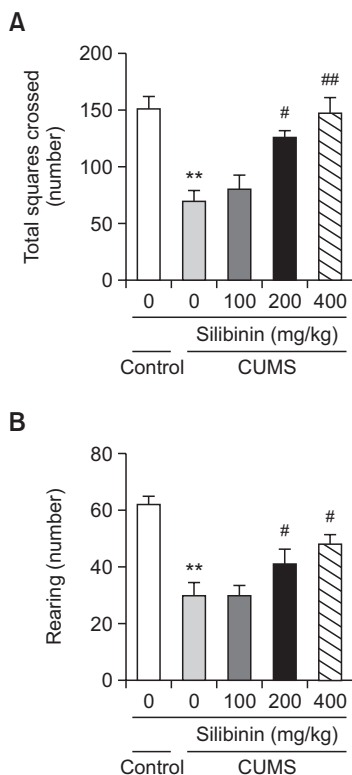


Fig. 1. Effects of silibinin on CUMS-induced depression-like behavior in the open field test. (A) The total number of squares crossed. (B) The frequency of rearing. All values are expressed as the mean \pm SEM ($n=12$ each), ** $p<0.01$, compared to the control group. # $p<0.05$, ## $p<0.01$, compared to the CUMS group.

the reaction.

5-HT or NE protein levels were quantified by 5-HT or NE ELISA kits (Shanghai Westang, China). Standards or samples (50 μ l) were added to each well of 96-well plates that were coated with an anti-mouse 5-HT or NE antibody. The plates were covered with the adhesive strip provided and incubated for 40 min at 37°C. After 5 washes, 90 μ l of TMB Substrate were added to each well of the plates before they were incubated for 20 min at 37°C and protected from the light. Finally, 50 μ l of Stop Solution was added to stop the reaction.

The concentrations of each sample were determined in duplicate. The optical density (OD, microplate reader set to 450 nm) of each well was determined within 30 min. BDNF, 5-HT and NE levels were reported in pg/mg protein. The total protein concentrations were determined by the BCA method.

Statistical analysis

The results were expressed as the means \pm SEM. Statistical comparisons between groups were made using the one-way analysis of variance (ANOVA) followed by the Dunnett's post hoc test. $p<0.05$ were recognized as statistically significant.

RESULTS

Effects of silibinin in the open field test

In the open field test, the total number of squares crossed

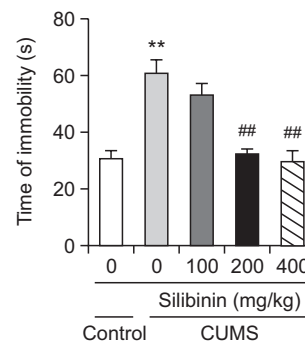


Fig. 2. Effects of silibinin on CUMS-induced depression-like behavior in the tail suspension test. All values are expressed as the mean \pm SEM ($n=12$ each), ** $p<0.01$, compared to the control group. # $p<0.05$, ## $p<0.01$, compared to the CUMS group.

and rearing in the last 5 min indicated depression-like behavior. Compared to the control group, the total number of squares crossed and rearing were significantly reduced in the CUMS group ($p<0.01$, Fig. 1A, 1B). The total number of squares in the CUMS+silibinin (200 and 400 mg/kg) groups were significantly increased compared to the CUMS group ($p<0.05$ for 200 mg/kg, $p<0.01$ for 400 mg/kg, Fig. 1A). The frequency of rearing in the CUMS+silibinin (200 and 400 mg/kg) groups was also significantly increased compared to that of the CUMS group ($p<0.05$ for 200 mg/kg and 400 mg/kg, Fig. 1B).

Effects of silibinin in the tail suspension test

In the tail suspension test, the results showed that the immobility time in the CUMS group was longer than the control group ($p<0.01$, Fig. 2), while 200 and 400 mg/kg silibinin significantly improved CUMS-induced depression-like behavior ($p<0.01$ for 200 mg/kg and 400 mg/kg, Fig. 2).

Effects of silibinin in the forced swimming test

In the forced swimming test, the results also suggested that immobility time in the CUMS group was longer than the control group ($p<0.01$, Fig. 3). Silibinin (200 and 400 mg/kg) also significantly ameliorated CUMS-induced depression-like behavior ($p<0.05$ for 200 mg/kg and $p<0.01$ for 400 mg/kg, Fig. 3).

Effects of silibinin on BDNF concentrations

There was a noticeable decrease in the levels of BDNF in the prefrontal cortex and hippocampus in the CUMS group compared to the control group ($p<0.01$, Fig. 4A). Silibinin treatment at 400 mg/kg significantly prevented the decrease in BDNF levels in the prefrontal cortex and hippocampus ($p<0.01$, Fig. 4a). Additionally, 200 mg/kg silibinin tended to increase BDNF levels in the prefrontal cortex, although the differences in the hippocampus were not significant (Fig. 4A).

Effects of silibinin on 5-HT and NE concentrations

5-HT concentrations in the prefrontal cortex and hippocampus tended to decrease in the CUMS group ($p<0.01$, Fig. 4B). After treatment with silibinin (200 and 400 mg/kg), the mice showed signs of improvement ($p<0.05$ for 400 mg/kg in prefrontal cortex, $p<0.05$ for 200 mg/kg and $p<0.01$ for 400 mg/kg in hippocampus, Fig. 4B). NE levels in the CUMS group were less than the control group ($p<0.01$, Fig. 4C). However,

silibinin treatment prevented the decrease of NE levels in both the prefrontal cortex and hippocampus ($p < 0.05$ for 200 mg/kg and $p < 0.01$ for 400 mg/kg, Fig. 4C).

DISCUSSION

Recent studies have demonstrated that long-term low intensity social pressure is a main cause of major depression (Salmon, 2001; Pryce *et al.*, 2005). Animal models are an effective means of studying the etiology and molecular pathogenesis of depression and antidepressant effects (Krishnan and Nestler, 2011). In our current study, the CUMS mice effectively simulated the depressive status as shown by decreased motor activity in the open field test and increased immobility time in the tail suspension test and forced swimming test, along with the reduction of BDNF, 5-HT and NE levels in the prefrontal cortex and hippocampus. However, silibinin administration attenuated the abnormalities indicative of depressive status in CUMS mice. Therefore, the present study revealed a prospective antidepressant property of silibinin, which may

be involved in changes in neuroplasticity and monoaminergic responses.

In the behavioral analyses, the mice exhibited depressive-like behaviors after 5 consecutive weeks of CUMS procedures. The results showed a notable decrease in the total number of squares crossed and the frequency of rearing in the open field test, as well as a significant increase in immobility time in the tail suspension test and forced swimming test. However, beginning at the 3rd week, daily silibinin administration by gavage at doses of 200 mg/kg or 400 mg/kg promoted antidepressant effects by attenuating behavioral abnormalities in CUMS mice. Moreover, 100 mg/kg silibinin has no apparent effects in our study.

BDNF is an essential neurotrophin that is involved in neuronal development and structural plasticity (McAllister *et al.*, 1999; Zhang *et al.*, 2010). BDNF is believed to underlie the pathophysiology and the therapeutic action of antidepressants upon binding to and activating tropomyosin-related kinase receptor B (TrkB) (Lee and Kim, 2010). In parallel with BDNF, increased mRNA expression of TrkB receptor in the hippocampus is also involved in the treatment of depression (Koike *et al.*, 2013). In previous studies, drugs that blocked the TrkB signaling pathway reversed the antidepressant-like effect of BDNF in rats (Shirayama *et al.*, 2002), and chronic antidepressant treatment enhanced hippocampal neurogenesis and increased BDNF and TrkB receptor expression in animal models (Li *et al.*, 2013). In the present study, we found that silibinin attenuated the levels of BDNF expression in CUMS mice. Silibinin administration at doses of 200 mg/kg and 400 mg/kg by daily gavage for 3 consecutive weeks significantly increased BDNF levels in the prefrontal cortex and hippocampus of CUMS mice, further indicating that BDNF may participate in the antidepressant-like effect of silibinin. We, therefore, speculate that the potential neuroprotective mechanism of silibinin likely occurs through activating the BDNF-TrkB pathway to produce protective effects in CUMS mice. However, this hypothesis requires further investigation for confirmation.

The neurotrophic hypothesis of major depressive disorder is accompanied by the monoamine theory (Maletic *et al.*, 2007), which postulates that low levels of monoamines are

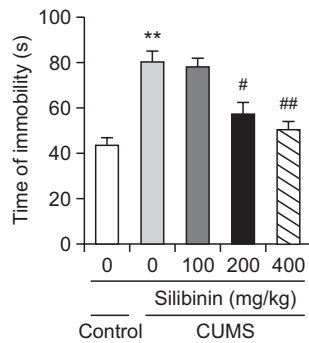


Fig. 3. Effects of silibinin on CUMS-induced depression-like behavior in the forced swimming test. All values are expressed as the mean \pm SEM ($n=12$ each), ** $p < 0.01$, compared to the control group. # $p < 0.05$, ## $p < 0.01$, compared to the CUMS group.

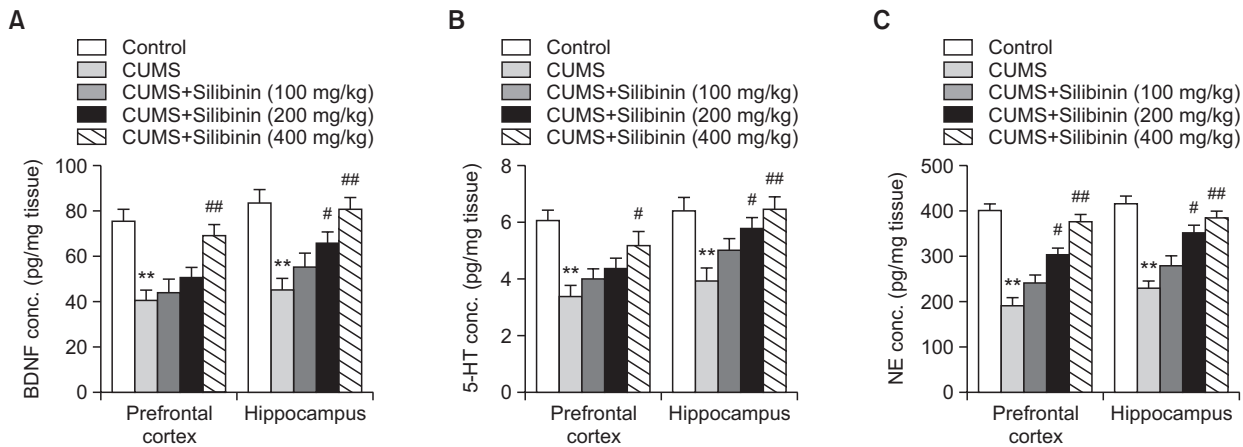


Fig. 4. BDNF, 5-HT and NE levels in the prefrontal cortex and hippocampus as measured by ELISA. (A) BDNF levels in the prefrontal cortex and hippocampus. (B) 5-HT levels in the prefrontal cortex and hippocampus. (C) NE levels in the prefrontal cortex and hippocampus. All values are expressed as the mean \pm SEM ($n=10$ each), ** $p < 0.01$, compared to the control group. # $p < 0.05$, ## $p < 0.01$, compared to the CUMS group.

involved in the incidence of depression, particularly 5-HT and NE (Blier and El Mansari, 2013; Huang *et al.*, 2014). Over the past few decades, new drug development in depression targeted the serotonergic system, more specifically, 5-HT reuptake (Maes, 2008). In the present study, we found that CUMS mice markedly decreased 5-HT and NE levels in comparison with the control group. Silibinin administration at doses of 200 mg/kg and 400 mg/kg significantly reversed these parameters in the prefrontal cortex and hippocampus, but 400 mg/kg seemed to be much more effective. These results suggested that the monoamine system may be involved in the antidepressant-like effects of silibinin, and the mechanism may be associated with the antioxidant and anti-inflammatory properties of silibinin. Some studies revealed that increased oxidative load is markedly related to the alterations in the central monoaminergic systems (Ahmad *et al.*, 2010). During the process of oxidative stress, excessive free radicals can cause oxidative damage to lipids, and further lead to neuronal injury or even apoptosis (Manzanero *et al.*, 2013). Some other studies noted that there is a bidirectional interaction between the monoamine system and the inflammatory system (Grippo and Johnson, 2009). Peripherally administered cytokines interacted with the monoamine metabolism and could activate the CNS inflammatory response in humans and further influenced the development of depression (Dantzer *et al.*, 2008; Raison *et al.*, 2009). Therefore, silibinin may protect the neurons of CUMS mice from monoamine reduction via its antioxidant and anti-inflammatory properties.

Lack of a positive control group is a limitation of the present study. Nevertheless, our findings still indicated that silibinin administration could significantly alleviate the CUMS-induced depressive-like behaviors, as well as affect neuroplasticity and neurotransmission in the prefrontal cortex and hippocampus. Moreover, we will carry out a separate but more extensive study that includes fluoxetine as a positive control group to further confirm these preliminary results and determine the underlying mechanisms.

In summary, silibinin administration at doses of 200 mg/kg and 400 mg/kg resulted in significant protective effects in the open field test, tail suspension test, and forced swimming test and increased the levels of BDNF, 5-HT and NE in the prefrontal cortex and hippocampus in CUMS mice. This study provides new insight into the antidepressant effects of silibinin, which may be regulated by improving neuroplasticity and neurotransmission in the CUMS induced depression mouse model.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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