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ORIGINAL ARTICLE

Berberine Ameliorates Chronic Unpredictable Mild Stress Induced Depression Like Behaviors and Elevating Kelch-like Erythroid Cell-derived Protein with CNC Homology-associated Protein 1-nuclear Factor (Erythroid-derived 2)-like 2 Antioxidant Signaling Pathways

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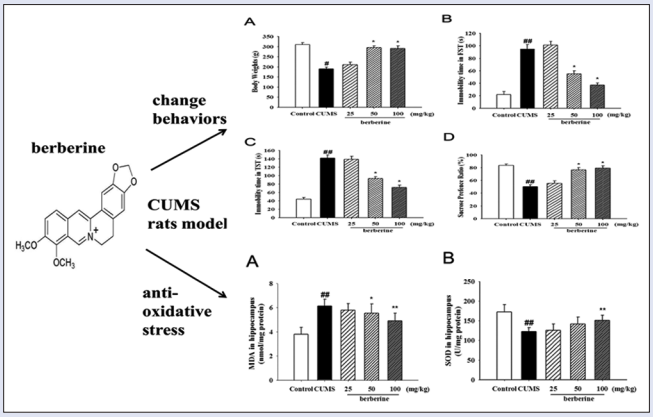
ABSTRACT

Background: Studies have reported that depression is associated with increased level of oxidative stress and accompanied with decreased Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1-nuclear factor (erythroid-derived 2)-like 2 (Keap1-Nrf2) signaling pathway. Berberine has been shown to possess properties on protection against neurodegenerative diseases, but its effects on chronic unpredictable mild stress (CUMS) induced depressive such as behaviors and Keap1-Nrf2 signaling pathway are unknown. **Objective:** This study was designed to evaluate the effect of berberine on CUMS induced depression animal model and investigate the underlying mechanisms. **Materials and Methods:** We established CUMS depressant rats model and treated CUMS rats with berberine. Sucrose preference test, forced swim test , and tail suspension test were used to measure behavioral changes. We used quantitative polymerase chain reaction and western blot to test the levels of cytokines in the hippocampus. **Results:** We found that CUMS rats displayed obvious depressive-like behaviors. Moreover, berberine treatment prevented depressive behaviors in CUMS rats accompanied with suppression of oxidative stress markers. Further experiments showed that berberine treatment up-regulated the expression of Keap1-Nrf2 antioxidant signaling pathway and its downstream neuroprotective factors. **Conclusion:** The present results suggested that treatment of berberine significantly ameliorated depressive-like behaviors in CUMS rats and enhanced Keap1-Nrf2 antioxidant signaling pathways in hippocampus.

Key words: Berberine, depressive-like behaviors, Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1-nuclear factor (erythroid-derived 2)-like 2 antioxidant signaling pathway

SUMMARY

- Berberine protects rats against chronic unpredictable mild stress-induced depressive-like behaviors
- Berberine declines oxidative stress in hippocampus of chronic unpredictable mild stress rats
- Berberine up-regulates Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1-nuclear factor (erythroid-derived 2)-like 2 antioxidant signaling pathway.



Abbreviations used: MDD: Major depressive disorder; CUMS: Chronic unpredictable mild stress; FST: Forced swim test; TST: Tail suspension test; Keap1: Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1; Nrf2: nuclear factor (erythroid-derived 2)-like 2; ARE: Antioxidant response elements; SOD: Superoxide Dismutase; MDA: Malonic dialdehyde; IMI: Imipramine.

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INTRODUCTION

Major depressive disorder (MDD) is the most prevalent psychiatric disease in the general population.[1] It is potentially life-threatening and the morbidity in people at the age of 19 is 25%,[2,3] <50% of the patients do not respond to the currently available medications and therapeutic effects.[4,5] The reason is that the molecular pathophysiology related to MDD is poor understood.

Studies support the hypothesis that MDD is driven by disruptions in prominent biological pathways and especially involved in the

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synaptic plasticity mechanism,[6-8] neuroimmune functions,[9] and the hypothalamus-pituitary-adrenal cortex hypothesis.[10] Dysregulations in biological stress systems, such as oxidative stress,[11] which are increasingly identified as potential underlying mechanism in MDD. Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1-nuclear factor (erythroid-derived 2)-like 2 (Keap1-Nrf2) signaling pathway, one of the most important defense mechanisms, protects cell against oxidative stress. Nrf2, a transcription factor, regulates several key downstream antioxidants expression by binding to antioxidant response elements (ARE) and ubiquitination regulated by Keap1. In repeated social defeat stress model, Nrf2 and Keap1 proteins showed decreased expression in the CA3, dentate gyrus of hippocampus and prefrontal cortex of mice.[12] Keap1-Nrf2 signaling pathway has been considered as a key pathway and promising therapeutic marker for depressive disease.

Berberine is an isoquinoline plant alkaloid, which can across the blood-brain barrier and could be transported into the neurons in a concentration-dependent and time-dependent manner.[13] Studies have suggested that berberine can produce a variety of biological effects in the central nervous system, and it has been shown to against various animal models of central nerve system-related disorders as a neuroprotective factor.[14] A study about neurodegenerative diseases has shown the protective effect of berberine.[15] Berberine has been demonstrated that it could significantly reverse depressive-like behaviors in depressive animal model,[16,17] and reverse the behavioral despair induced by reserpine (Kulkarni and Dhir, 2008). These researches suggested that berberine might possess antidepressant effect, which still need explore on spontaneous animal models of depression and study the potential mechanism of it.

Recently, we found that berberine showed the antidepressant-like effects in chronic unpredictable mild stress (CUMS) rats, and this effect might be mediated partially by reducing oxidative stress through elevating Keap1-Nrf2 pathway levels in CUMS rat hippocampus, which led to the change of depressive-like behaviors.

MATERIALS AND METHODS

Animals

All protocols were performed in accordance with the Guidelines of China Three Gorges University, and all procedures involved in the use of laboratory were approved by the Animal Ethics Committee of our institution. SD rats (8–10 weeks age, 200–250 g, male) were both purchased from the Animal Center of China Three Gorges University. All animals were housed under standard conditions with controlled temperature of 23°C ± 3°C, humidity of 50% ±10%, and a 12 h light-dark cycle with free access to water and food. Berberine (NORTHEAST. PHARM, Shenyang, China) was dissolved in drinking water at a concentration of 25, 50 and 100 mg/kg. Imipramine hydrochloride was purchased from the Solarbio company (Wuhan, China). After adaptation of 1 week, healthy rats were randomly divided into six groups: control group (n = 8), model group (n = 8), 25 mg/kg group (n = 8), 50 mg/kg group (n = 8), 100 mg/kg group (n = 8), and imipramine (IMI) group 15 mg/kg (n = 8). All rats were administered drug orally for 21 days.

Chronic unpredictable mild stress model

The rats were subjected to CUMS for 5 weeks. The procedure was as described previously,[18,19] with slight modifications. In brief, CUMS was included exposure to a variety of unpredictable stressors: Two 16-h period of water deprivation, two periods of continuous overnight illumination, two periods (7 and 17 h) of 45 cage tilt, two periods (7 and 17 h) in a soiled cage (100 ml water in sawdust bedding), two periods (2 h) of dark in the day time, two periods (3 and 5 h) of noise (30 dB, 10kc/s), and two periods of 3 min cold swim at 4°C (after which they were toweled dry). The 14 stressors were administered in a semirandom manner so that they were unpredictable for the subjects. In this way, these stressors were scheduled randomly over a 1-week period and repeated throughout the 5-week experiment. On average, two stressors were applied daily [Table 1]. Control animals were housed in a separate room and had no contact with the stressors and stressed rats. After 5 weeks of CUMS, the rats were subjected to the behavioral tests in the following order (the least stressful test was performed first): sucrose preference test (SPT), force swimming test (FST), tail suspension test (TST) with a 2-day interval between each test. Each subject was used only once per behavioral test and all animals were tested in a predetermined sequence, which was maintained constant among tests. The observers were blinded to the experiment groups for all of the behavioral tests.

Sucrose preference test

Rats of both ages were simultaneously given a free choice between two bottles, one with 1% sucrose solution and the other with tap water, for 8 h, between 09:00 and 17:00. The beginning of the test started with the onset of the dark (active) phase of the animals' cycle. No previous food or water deprivation was applied before the test. After stress or berberine treatment, rats were deprived of water and food for 23 h. SPT was conducted in which rats were housed in individual cages and were free to access to two bottles containing 100 ml of sucrose solution (1% w/v) and 100 ml of water. After 1 h, the volumes of consumed sucrose solution and water were recorded and the sucrose preference was calculated as the sucrose preference (%) = sucrose consumption/(sucrose consumption + water consumption).[20]

Force swimming test

The forced swimming test was carried out on rats individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 15 cm of water at 25°C ± 1°C; Each mouse was judged to be immobile when it ceased to struggle and remained floating motionless in the water, making only those movements necessary to keep its head above water. The duration of immobility was recorded. Decrease in the duration of immobility during the FST was taken as a measure of antidepressant activity.[21] The total duration of immobility during the 6-min test was recorded. After the initial 2 min of vigorous activity, rats were observed for immobility for next 4 min. An animal was considered to be immobile whenever it remained floating passively in the water in a slightly hunched but upright position, its nose above the water surface.

Table 1: Schedule of stressor used in the 35 days of a chronic unpredictable mild stress procedure

Stressors	Duration	Day
Water deprivation	16 h	Monday
45°cage tilt	17 h	
Overnight illumination	12 h	Tuesday
Soiled cage	7 h	
Water deprivation	16 h	Wednesday
Dark in the day time	2 h	
Noise (30dB, 10kc/s)	3 h	Thursday
Cold swim at 4°C	3 min	
Overnight illumination	12 h	Friday
45°cage tilt	7 h	
Soiled cage	17 h	Saturday
Noise (30 dB, 10 kc/s)	5 h	
Dark in the day time	2 h	Sunday
Cold swim at 4°C	3 min	

A schedule illustration of the experimental procedure used in this study

Tail suspension test

Rats were submitted to TST at 24 h after stress or berberine treatment. The total duration of immobility induced by tail suspension was measured according to the method.[22] Rats both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The time during which rats remained immobile was quantified during a test period of 6 min. Rats were considered immobile only when they hung passively and completely motionless.

Oxidative stress markers and neuroprotective factors content test

The activity and content of oxidative stress markers superoxide dismutase (SOD), malondialdehyde (MDA) and neuroprotective factors glutathione (GSH), glutathione peroxidase (GPx) activity were test according to the instruction (Nanjing Jiancheng).

Reverse transcription polymerase chain reaction

Polymerase chain reaction (PCR) analysis was performed on Real time quantitative PCR Instrument MX 3005p (Agilent Technologies Co., America) using One Step SYBR® PrimeScript™ RT-PCR Kit (Takara, Japan). The primers used for the PCR amplification were synthesized by Invitrogen (China). All reactions were running triplicate. The relative expression levels of nicotinamide adenine dinucleotide phosphate dehydrogenase (quinone) 1 (NOQ1) and γ-glutamyl cysteine synthetase (γGCS) were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression level. Fold changes relative to control samples were determined: NOQ1 (Forward: 5'-CAGAAACGACATCACAGGGGA-3', Reverse: 5'-AGCACTCTCTCAAACCAGCC-3'), γGCS (Forward: 5'-CAGAAACGACATCACAGGGGA-3', R: 5'-AAACCTGATGGC ATTGTGAGAC-3'), GAPDH (Forward: 5'-TTGTCAAGCTC ATTTCCTGG-3', Reverse: 5'-TGTGAGGAGGGGAGATTCAG-3').

Western blot

Anti-Keap1, anti-Nrf2 antibody was purchased from Proteintech Biotechnology (Wuhan, China), anti-β-actin antibody (Santa Cruz, USA) was used as an internal control antibody. Proteins of tissue were extracted with SDS lysis buffer (Beyotime, China), and separated by SDS-PAGE gel. Subsequently, protein samples were transferred to nitrocellulose membrane (NC; Millipore, USA). Membranes were probed with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detected by Gel Imaging System/UV Light Boxes (Clinx Science Instruments Co. China).

Statistical analyses

All numerical data are expressed as the mean ± standard error of mean. Statistical differences among groups were analyzed using Student's *t*-test and one-way ANOVA with *post hoc* correction. Statistics significance was calculated using version SPSS 13.0. The value of $P < 0.05$ was considered as statistically significant.

RESULTS

Berberine in different concentration prevented the depressive-like behaviors

The design and process of present experiment are shownin Figure 1. Berberine in different concentration 25, 50, and 100 mg/kg were administered to the CUMS rats for consecutive 21 days [Figure 1]. In CUMS rats, the bodyweights [Figure 2a] and sucrose consumption [Figure 2d] of CUMS rats were declined. The immobility times of FST [Figure 2b] and TST [Figure 2c] were increased. After 21 days treatment, we found that the concentration of 100 mg/kg berberine, IMI (15 mg/kg) reversed above depressive-like behaviors and increase bodyweights of model group rats.

Berberine decreased oxidative stress damage in hippocampus

MDA content and SOD activity in hippocampus of rats are used to illustrate oxidative stress state. MDA content in hippocampus of rats was increased and SOD activity was the opposite. The level and activity of two cytokines in berberine and IMI treatment group were corrected into normal degree [Figure 3a and b].

Berberine up-regulated Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1-nuclear factor (erythroid-derived 2)-like 2 signaling pathway in hippocampus

To ensure whether berberine reversed the depressive-like behaviors through Keap1-Nrf2 signaling pathway, western blot were used to assay Keap1 and Nrf2 protein expressions. Levels of Keap1 and Nrf2 protein in the hippocampus of CUMS group rats were significantly lower than those of control group. As shown in Figure 4a and b, berberine at the concentration of 50, 100 mg/kg and IMI (15 mg/kg) partly increased the protein expressions of Keap1 and Nrf2 in hippocampus of CUMS rats.

Berberine increased glutathione peroxidase activity and glutathione content in hippocampus

GSH content and GPx activity are considered to be important indicator

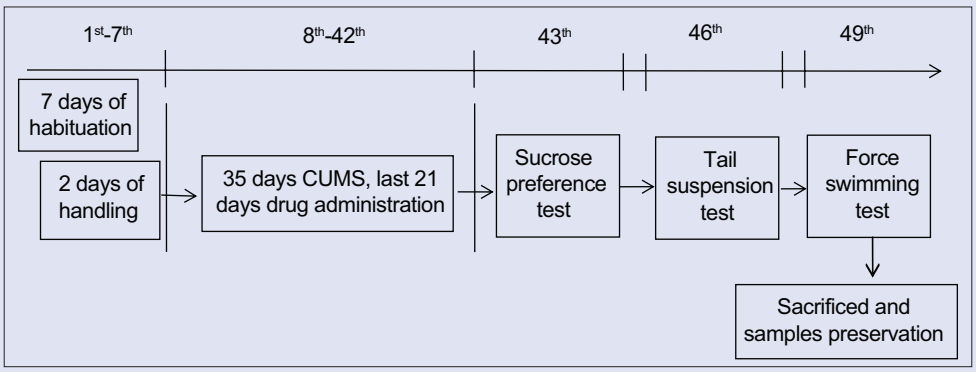

Figure 1: Experiment progress of berberine treatment in CUMS model. CUMS: Chronic unpredictable mild stress

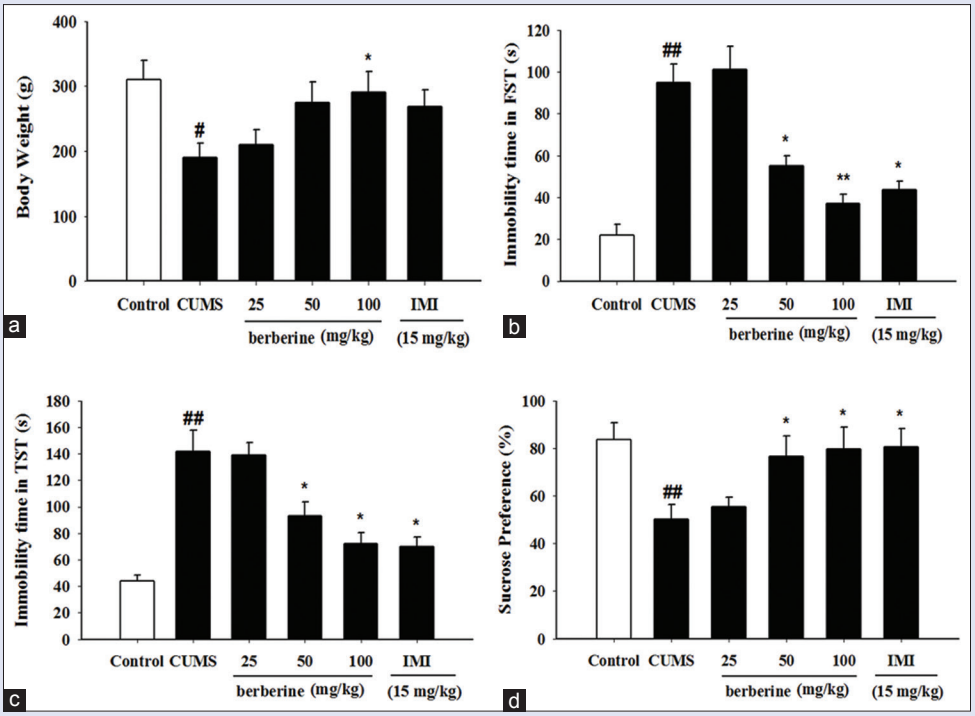

Figure 2: Effects of berberine on the body weight (a), immobility time in FST (b), TST (c), and sucrose preference (d) in CUMS rats. Data were expressed as the mean ± standard error of mean ($n = 8$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus control, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus CUMS. CUMS: Chronic unpredictable mild stress; FST: Force swimming test

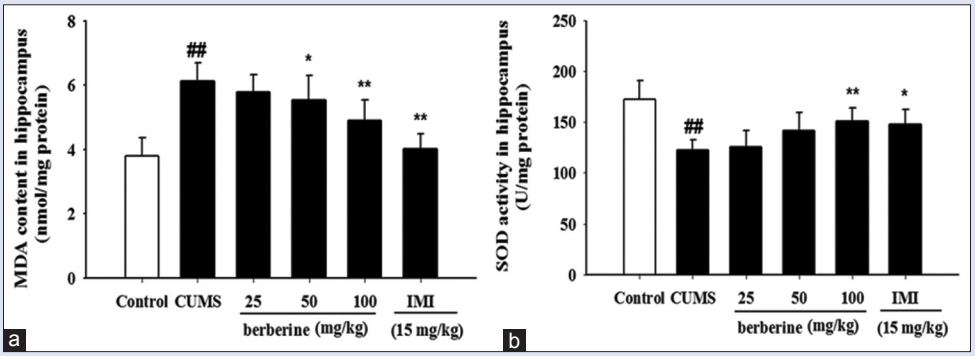

Figure 3: Effects of berberine on MDA content (a) and SOD activity (b) in hippocampus of CUMS rats. Data were expressed as the mean ± standard error of mean ($n = 4$). $^{\#\#}P < 0.01$ versus control, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus CUMS. CUMS: Chronic unpredictable mild stress; MDA: Malondialdehyde; SOD: superoxide dismutase

of antioxidant capacity. The GSH content and GPx activity were lower in the hippocampus of CUMS rats but were significantly improved by berberine and IMI treatment [Figure 5a and b].

Berberine increased nicotinamide adenine dinucleotide phosphate dehydrogenase (quinone) 1 and γ-glutamyl cysteine synthetase messenger RNA expression in hippocampus

NOQ1 and γGCS are important downstream targets of Keap1-Nrf2 signaling pathway. CUMS rats displayed lower mRNA expressions of NOQ1 and γGCS in the hippocampus compared with those of control group. Berberine and IMI treatment significantly increased NOQ1 and γGCS messenger RNA (mRNA) expressions in hippocampus of CUMS model group [Figure 6a and b].

DISCUSSION

A line of evidence demonstrates that berberine is associated with anti-depression effect,[23] and the mechanisms are involved in various targets and pathways. In our study, CUMS depressive animal model was used to explore the anti-depressive effect and mechanism of berberine. We found that compared with control rats, model group rats displayed obvious depressive-like behaviors, including sucrose preference decreased, immobility times in FST and TST prolonged. At the concentration of 100 mg/kg berberine, the depressive-like behaviors of CUMS rats were reversed. Moreover, oxidative stress markers were increased in the hippocampus of CUMS rats. However, Keap1-Nrf2 signaling and downstream factors, including NQO1 andγGCS were down-regulated. After berberine treatment for consecutive 21 days, all these cytokines were normalized.

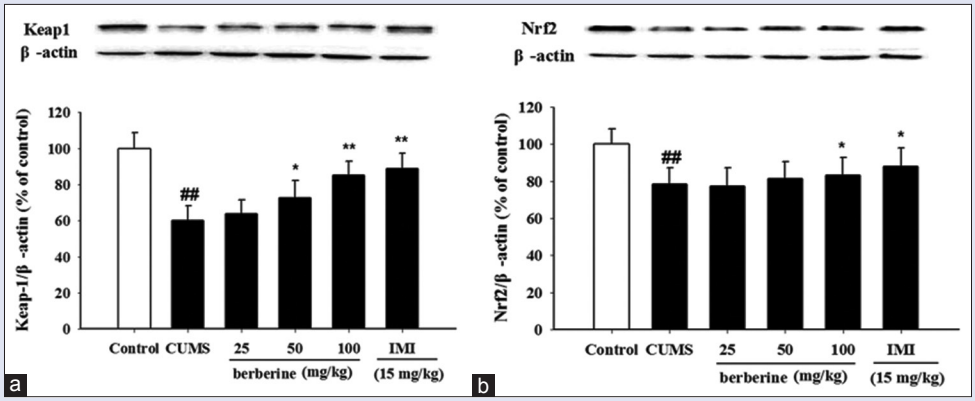

Figure 4: Effects of berberine on the Keap1 (a) and Nrf2 (b) protein level in hippocampus of CUMS rats. Data were expressed as the mean ± standard error of mean ($n = 4$). ##$P < 0.01$ versus control, *$P < 0.05$ and **$P < 0.01$ versus CUMS. CUMS: Chronic unpredictable mild stress; Keap1: Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1, Nrf2: Nuclear factor (erythroid-derived 2)-like 2

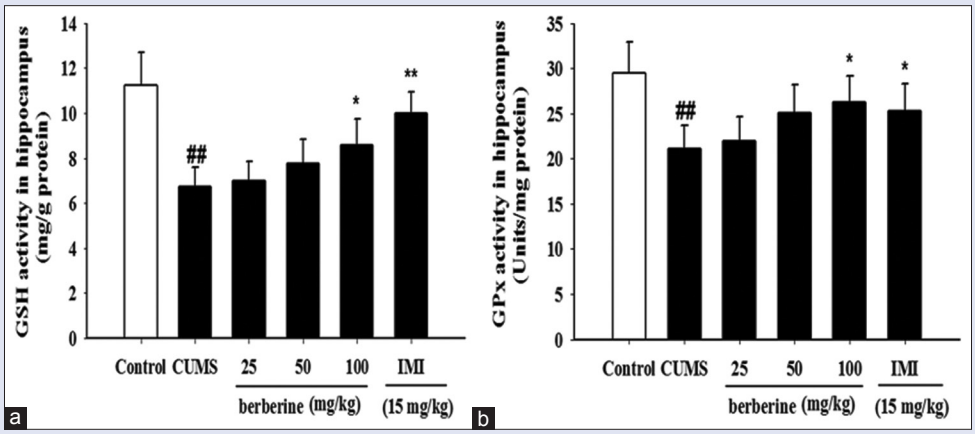

Figure 5: Effects of berberine on GSH (a) and GPx (b) activity in hippocampus of CUMS rats. Data were expressed as the mean ± standard error of mean ($n = 4$). ##$P < 0.01$ versus control, *$P < 0.05$ and **$P < 0.01$ versus CUMS. CUMS: Chronic unpredictable mild stress; GSH: Glutathione; GPx: Glutathione peroxidase

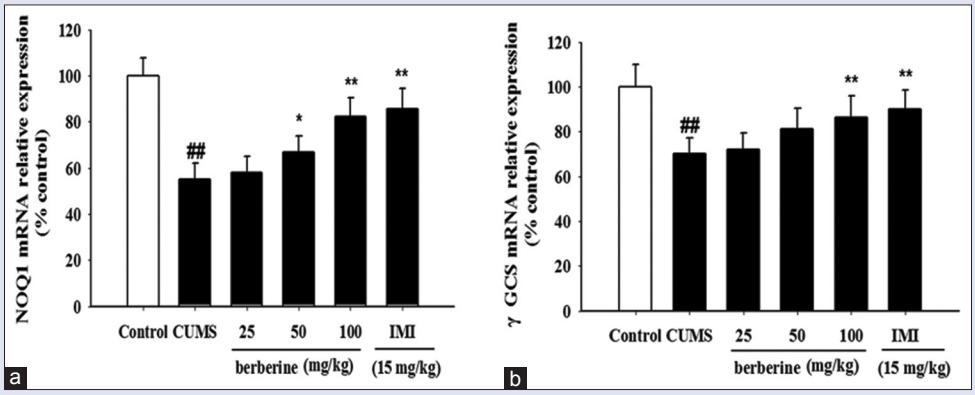

Figure 6: Effects of berberine on the NOQ1 (a) and γGCS (b) messenger RNA level in hippocampus of CUMS rats. Data were expressed as the mean ± standard error of mean ($n = 4$). ##$P < 0.01$ versus control, *$P < 0.05$ and **$P < 0.01$ versus CUMS. CUMS: Chronic unpredictable mild stress, NOQ1: Nicotinamide adenine dinucleotide phosphate dehydrogenase (quinone) 1, γGCS: γ-glutamyl cysteine synthetase

The present study *in vivo* was conducted in chronic unpredictable mild stress animal model, which can reflected stress-linked human conditions. SPT has been interpreted in the literature as an index of anhedonia-like behavioral change.[24] Anhedonia, one of the core criteria for major depression diagnosis, has been defined as a decrease in responsiveness to rewards reflecting by a reduced intake of palatable sweet solutions. The immobility of TST represents a failure of persistence in escape-directed behavior, which may be analogous to the clinical observations that depressed patients often lack sustained expenditure of effort reflected in a pronounced psychomotor impairments.[25] In the present study, we

found that CUMS rats displayed sucrose consumptions reduced and elevated immobility times in FST and TST. In addition, bodyweights of CUMS rats were also a little decreased at the end of model established. These results suggested that after 5 weeks of unpredictable stress, rats have shown obvious depressive-like condition which similar to clinical patients. Moreover, we also found that the expressions of Keap1-Nrf2 signaling and downstream factors NOQ1 and γGCS, were down regulated, oxidative stress markers increased, which further suggested that the CUMS rat is a suitable animal model to study the mechanism and assess the treatment effect of depressive disease prevention. Using CUMS rat model, we evaluated the effect of berberine on anti-depression. Recent results have shown that berberine decreased immobility times in FST and TST. Further, sucrose consumptions and bodyweights of CUMS rats were also increased after berberine treatment. All the above indicate that berberine is a promising candidate for protection against unpredictable stress induced depressive disease.

Central nerve system, especially brain tissue, was vulnerable to oxidative stress for its special construction and constituent. Moreover, after oxidative stress damage, it is hard for neurons to regenerate for its unrenewable feature. The activity of SOD and content of MDA in hippocampus were used to assess the level of reactive oxygen species. Keap1-Nrf2 signaling pathway plays a central role in cellular defense against oxidative.[26,27] Under normal conditions, Nrf2 stores in the cytoplasm with inactive state and repressed by Keap1, which was an adaptor protein for the degradation of Nrf2. During oxidative stress status, Nrf2 is de-repressed by Keap1 and activated, subsequently recognizes ARE and binds it to start expression of downstream neuron protection factors, including NOQ-1and γ-GCS. After Nrf2 activation, GSH, an important nerve protection factor, secretes from astrocyte. GSH content can be used as an important indicator to evaluate the antioxidant capacity of cell. The interest fact is that Keap1-Nrf2 signaling appears to decline with depressive disease because of long time chronic stress lead to dysregulation of oxidative stress responses.[12] In line with previous research, recent data showed that the level of Keap1-Nrf2 signaling pathway in hippocampus in CUMS group rats appeared to decline after chronic stress. MDA levels in hippocampus of CUMS group rats were significantly higher and SOD, GSH, GPx activity and Nrf2 downstream targets NOQ-1, γ-GCS were significantly lower in the hippocampus of CUMS group rats compared to control group. In all, our recent results suggested that chronic stress might induce oxidative stress in hippocampus, meanwhile Keap1-Nrf2 signaling pathway levels decreased and lead to an attenuated antioxidant defense.

Berberine reverses the depressive-like behaviors because the interaction with brain-derived neurotrophic factor, N-methyl-D-aspartate receptor, dopamine receptors,[28] this indicates that the target of berberine is various. In the present study, our results showed that chronic berberine administration reduced the content of MDA and increased activities of SOD, GPx and GSH level in hippocampus of CUMS rats by enhancing Keap1-Nrf2 antioxidant signaling pathway. Previous researches showed that berberine engaged in multiple pharmacological activities by protection cell against oxidative stress induced damage via upregulation of Nrf2 expression.[29,30]The study results were consistent with these studies. Thus, we speculated that the potential mechanism for berberine to protect against chronic unpredictable mild stress induced depressive like behaviors might be attributed to the capacity that enhancing the cell antioxidant defensive system.

In the recent study, we found that imipramine, the first line medicine for clinical depression treatment, also reduced oxidative stress and elevated Keap1-Nrf2 signaling pathway. Our results revealed a new mechanism of IMI in anti-depression action.

CONCLUSION

This study proved that CUMS rats, which displayed increasing oxidative stress status and reducing Keap-Nrf2 signaling pathway, were animal model simulating human major depressive disease. On the basis of CUMS model, we found that treatment of berberine significantly ameliorated depressive-like behaviors, which, in part, might be associated with enhancing of Keap1-Nrf2 antioxidant signaling pathways. Taken together, our results suggested that berberine might be a promising molecule for the prevention of major depression.

Acknowledgements

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Nil.

Conflicts of interest

There are no conflicts of interest.

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