

Original Research Article

Mitigation of chronic unpredictable stress-induced cognitive deficits in mice by *Lycium barbarum* L (Solanaceae) polysaccharides

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Abstract

Purpose: To investigate the neuroprotective effects of *Lycium barbarum* polysaccharide (LBP) against concomitant cognitive dysfunction and changes in hippocampal CREB-BDNF signaling pathway in chronically unpredictable stressed mice.

Methods: The mice were subjected to different unpredictable stressors for a period of 4 weeks. Behavioral tests, including open field (OFT) and Morris water maze (MWM) tests were used to evaluate pharmacological effects. Serum corticosterone levels, protein expression level of BDNF and pCREB/CREB in hippocampus were assessed by ELISA, Western blot and immunohistochemistry methods, respectively. Morphological changes in pyramidal neurons in the hippocampus were studied by Nissl staining.

Results: LBP improved mice performance in MWM, indicating that it reversed chronic unpredictable stress (CUS)-induced cognitive deficits. LBP treatment reduced serum corticosterone levels and prevented neuron loss in the hippocampus. It maintained expression levels of BDNF and phosphorylation of CREB in hippocampus during CUS procedure.

Conclusion: *Lycium barbarum* polysaccharide protects CREB-BDNF signaling pathway in hippocampus and relieves CUS-induced cognitive deficits. These results suggest that *Lycium barbarum* polysaccharides is potentially an alternative neuro-protective agent against stress-induced psychopathological dysfunction.

Keywords: *Lycium barbarum*, Polysaccharide, Chronic unpredictable stress, Cognitive deficits, Brain-derived neurotrophic factor, Calcium/cyclic-AMP responsive binding protein

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INTRODUCTION

Lycium barbarum (wolfberries, Solanaceae) is used in traditional Chinese medicine for treating diminished visual acuity, dry cough, abdominal pain, headache, infertility, and fatigue [1]. *Lycium barbarum* polysaccharides (LBP), the polysaccharide extract from *Lycium barbarum* fruit, contains protein, antioxidants, galacturonic

acid, neutral sugars, arabinose, galactose, rhamnose, xylose, glucuronic acid, mannose and glucose [1]. In recent years, many pharmacological effects of LBP have been reported. LBP has hypoglycemic and hypolipidemic effects, and it could be used for treating type 2 diabetes [2] and cancer [3]. LBP improves sexual behavior and increases

neurogenesis [4], as well as Alzheimer's disease (AD), colitis, fatigue, glaucoma and stroke [5].

Pre-clinical and clinical studies on stress have demonstrated a wide range of neurochemical and morphological alterations that could contribute to psychopathological derangements such as cognitive disorders [6,7]. Chronic unpredictable stress (CUS), which is one of the extensive used rodent models in stress related mental disorders such as anxiety and depression [8]. Exposure to CUS has been shown to induce cognitive deficits [7], reduce neurotrophic factors, increase corticosterone levels and even decrease the number of neurons and glia in hippocampus and other cerebral regions [6].

Brain-derived neurotrophic factor (BDNF), the widely spread neurotrophic factor in the brain, has extensively been attributed to stress and cognitive deficits related synaptic plasticity in several brain regions [9]. Calcium/cyclic-AMP responsive binding protein (CREB), the upstream molecule of BDNF, plays an important role in research on memory system and adult hippocampal neurogenesis [10]. Studies have shown that dysregulation of the CREB-BDNF cascade is involved in mental dysfunction in stress procedure, and that potentiation of the CREB-BDNF signaling pathway could be a specific marker of restoration in stress-induced cognitive deficits [9].

Behavioral and morphological alterations induced by CUS can be reversed by treatment with most current anti-depressants such as fluoxetine [11]. Fluoxetine treatment can stop the long-term stress induced depression and spatial cognitive dysfunction [12]. In addition, a single injection of fluoxetine can reverse stress-induced damage in LTP at synapses from the hippocampus to prefrontal cortex in rats [13,14]. However, current antidepressants are associated with adverse reactions. The use of complementary and alternative medicine for management of stress-induced psychopathological dysfunction has continued to receive serious attention. New healing approaches and different measures to succeed the impact of chronic stress are necessitated, and analysis of selected functional foods can provide important leads.

The goal of the current study was to investigate whether LBP can produce beneficial effects against concomitant cognitive dysfunction in chronically stressed mice, and the role of LBP in protection of hippocampal neurons from damage. The role of LBP in regulation of serum corticosterone levels, and up-regulating effect of

CREB-BDNF signaling pathway in hippocampus during CUS were also investigated.

EXPERIMENTAL

Materials

Lycium barbarum polysaccharide (LBP) was purchased from Wolfberry Co. Ltd (Ningxia, China, batch no. 64WFB140301P). Fluoxetine was purchased from Eli Lilly & Co (Indianapolis, USA).

Animal handling and treatment schedules

All the animal procedures were performed in compliance with the Institute of Animal Care and Use Committees of Ningxia Medical University. A total of 90 adult male ICR mice weighing 20 ± 2 g, were provided by Experimental Animal Center of Ningxia Medical University (no. SCXY 2005-0001). The mice were accommodated 5 per cage and acclimatized for 3 days in a room with 12h:12h light: dark cycle; temperature of 22 ± 2 °C and 40 ± 10 % relative humidity.

After acclimatization, the mice were randomly assigned to six groups with 15 mice per propylene cage: Normal group (served as negative control with no stressor and normal feeding in a separate room); Vehicle group (served as CUS depression model group): stressor plus 0.9 % NaCl); LBP groups (stressor plus separate LBP administration at a doses of 40, 125 and 400 mg/kg)[15] and fluoxetine group (acted as positive control): stressor plus 10 mg/kg fluoxetine). The stress-exposed groups were subjected to repeated drug treatment once daily between 13:00 and 14:00pm by intragastric administration 1h before stress exposure. In this experiment, 24 mice died during the CUS procedure, leaving about 10 mice in each group. The flow chart of the experiment is shown in Figure 1 (a). This research was supported by the Animal Ethical Committee of Ningxia Medical University (approval no. 2016010) according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [16].

Chronic unpredictable stress procedures

During the experiment, mice in the normal group were left undisturbed (except regular cage cleaning and feeding) in a separate room. The mice in the other groups exposed to CUS were housed individually. The CUS procedure was carried out according to the method as described previously [17,18], but with slight modifications as outlined in Supplementary Information.

Behavioral analysis

Open-field test and Morris water maze test were carried out as previously described [18,19] and the details are provided in Supplementary Information.

Measurement of serum corticosterone level

Blood samples were collected then centrifuged and stored at -80°C until analysis. Plasma corticosterone was measured by ELISA kit (Abcam, Cambridge, UK) and Multiskan Go (Thermo Fisher scientific, Vantaa, Finland).

Histochemical analysis via Nissl staining

The procedure used is described in Supplementary Information. The number of stained cells in each field was counted at $\times 400$ magnification (BX-51, Olympus, Japan), and presented as percentage of normal group per high-power field.

Immunohistochemical studies of p-CREB and BDNF

Sections were stained with BDNF (polyclone, 1:100, Abcam); phospho-CREB (p-CREB, Ser133, monoclonal, 1:800, Cell Signaling). The images were acquired at $\times 400$ magnification (BX-51, Olympus, Japan). Quantitative histological evaluations of related protein expression mean density in hippocampus were performed by Image-Pro plus 6.0 software (Media Cybernetics Inc. MD, USA).

Western blotting analysis

Western blot was carried out using assay kits in line with the procedure described in Supplementary Information. Image acquisition was processed by Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, MA, USA) and ChemiDoc XRS + imaging system (BIO-RAD, CA, USA). The Mean Gray Value of bands was determined by Image J software (National Institute of Health, MD, USA).

Statistical analysis

All analyses were performed by GraphPad InStat 3.0 and GraphPad Prism 5.0 software (GraphPad Software Inc., USA). Data are presented as mean $\pm$ SEM. Differences between mean values were evaluated by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Chronic LBP treatment enhanced exploration of new environment by mice

The OFT results are shown in Figure 1 (b). CUS significantly decreased the number of squares entered by mice ($n = 10$, $p < 0.01$, vs. normal), indicating that CUS procedure decreased interest of mice in exploring new environment. Post hoc analysis revealed that this deficit was relieved by LBP treatment at the doses of 40 mg/kg ($n = 10$, $p < 0.05$, vs. vehicle), 400 mg/kg ($n = 10$, $p < 0.01$, vs. vehicle) and 10mg/kg doses of fluoxetine ($n = 10$, $p < 0.01$ vs. vehicle). These data confirm that CUS modeling was successful and indicate that LBP treatment enhanced the interest of the mice in exploring new environment.

LBP treatment reversed CUS-induced spatial learning and memory damage in mice

As shown in Figure 2 (b-d), CUS induced significant damage in spatial learning of mice (escape latency, $n = 8$, $p < 0.05$, vs. normal) and memory (percentage of distance in target quarter; $n = 8$, $p < 0.01$, vs. normal; percentage of time spent in target quarter; $n = 8$, $p < 0.01$, vs. normal). Furthermore, the locus diagram shown in Figure 2 (a) indicates that the swimming route of mice in the vehicle group was completely random. Figure 2 (b) shows that LBP reversed the spatial learning damage induced by CUS at the dose of 400 mg/kg by significantly decreasing escape latency. Results of Probe Trials are shown in Figure 2 (c) and Figure 2 (d). LBP reversed the memory damage induced by CUS at a dose of 400 mg/kg by significantly increasing the percentage of distance traveled in target quarter ($n = 8$, $p < 0.01$ vs. vehicle); and the percentage of time in target quarter ($n = 8$, $p < 0.01$, vs. vehicle). These results demonstrate LBP restored CUS-induced spatial learning and memory damage, and suggest that the cognitive deficit induced by CUS could be reversed by LBP treatment.

Chronic LBP treatment reduced serum corticosterone levels in CUS mice

As shown in Figure 1 (c), the level of serum corticosterone was significantly higher in stressed mice than normal mice (Figure 1 (c). $n = 10$, $p < 0.01$). Post hoc analysis showed that 400 mg/kg treated LBP had significantly decreased the serum corticosterone levels ($n = 10$, $p < 0.01$). These results demonstrate LBP reversed the impairment of feedback regulation of the HPA axis induced by CUS.

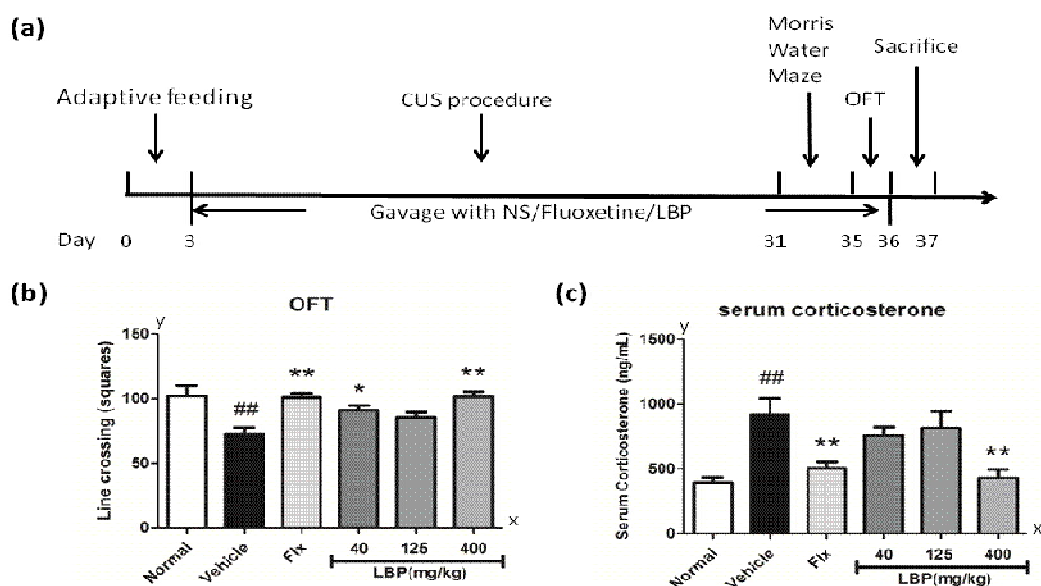


Figure 1: Effect of LBP relieve serum corticosterone level.

Note: In addition of Normal control group, Mice were exposed to CUS for 4 weeks and received a daily gavage (i.g) of saline (vehicle), Fluoxetine (Flx; 10 mg/kg) or LBP (40, 125, 400 mg/kg) during this 4 weeks. The success of CUS modeling was confirmed by OFT which were conducted 1h after the gavage in the 35th day. (a) The flow chart of the experiment. (b) LBP increased the line crossing in the OFT. (c) LBP relieves the CUS-induced serum corticosterone increased. The data are expressed as means + SEM (n = 10); *p < 0.05, **p < 0.01, significantly different from Vehicle; ^{##}p < 0.01, significantly different from Normal; one-way ANOVA followed by Dunnett test

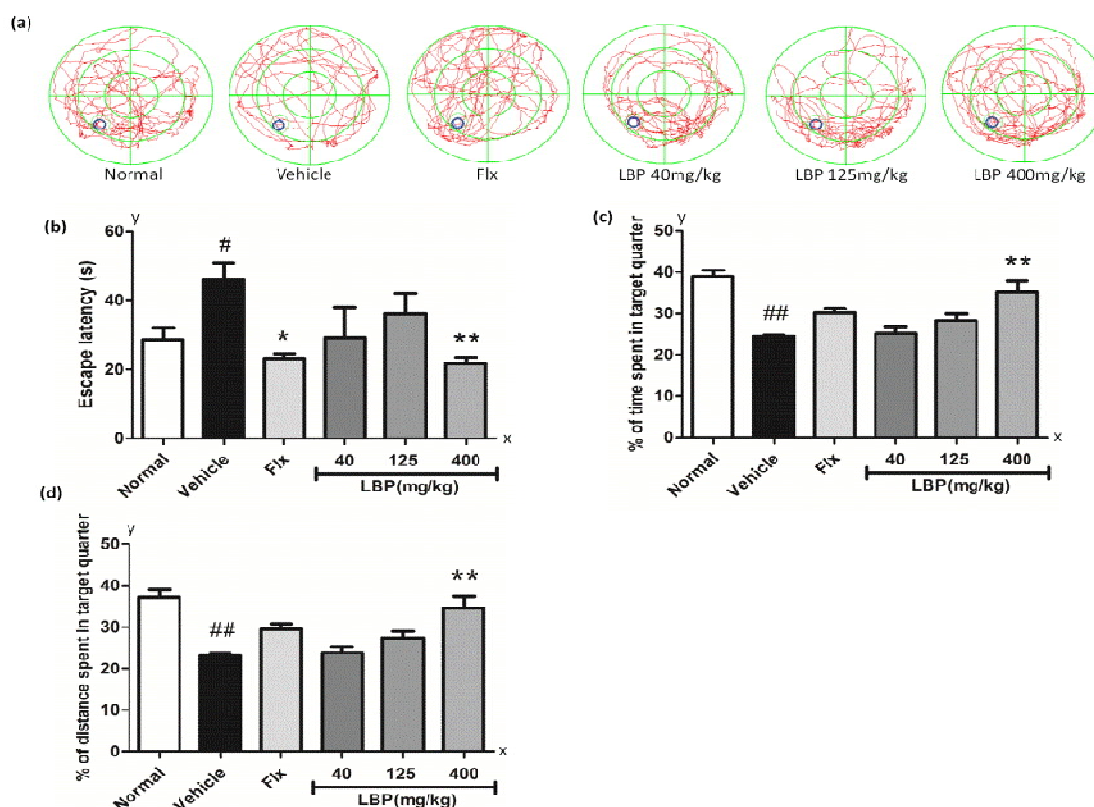


Figure 2: LBSP treatment reversed CUS-induced spatial learning and memory damage in MWM.

NOTE: (a) The probe tracks of Probe Trials. (b) LBP decreased the escape latency in learning procedure. (c) LBP increase the percentage of time spent in target quarter in Probe Trials. (d) LBP increased the percentage of traveled distance in target quarter in Probe Trials. These data are represented as means + SEM (n = 8). *p < 0.05, **p < 0.01, significantly different from vehicle; [#]p < 0.05, ^{##}p < 0.01, significantly different from normal; one-way ANOVA followed by Dunnett test.

LBP treatment relieved CUS-induced histomorphological changes in the hippocampus

Compared with normal pyramidal cells, as shown in Figure 3(a) and Figure 3(b), the CUS-induced morphological damage in the hippocampal neurons was obvious in the CA1 region ($n = 6$, $p < 0.05$, vs. normal). But, the post hoc analysis confirmed that the neuropathological deficits in CA1 region were significantly ameliorated by LBP (125 and 400 mg/kg) and Fluoxetine ($n = 6$, $p < 0.01$, vs. vehicle). These results demonstrate CUS causes severe loss of pyramidal neurons in the hippocampus, and that LBP treatment could relieve these neuronal losses.

LBP treatment restored BDNF signaling pathway in hippocampus of CUS mice

As shown in Figure 4 (a) and Figure 4 (b), unpredictable stress significantly attenuated the expression of hippocampal BDNF protein levels ($n = 6$, $p < 0.05$ vs. normal) in Western blotting studies, but this effect was reversed by fluoxetine ($n = 6$, $p < 0.01$ vs. vehicle) and LBP at a dose of 400 mg/kg ($n = 6$, $p < 0.01$ vs. vehicle). Moreover, similar results were seen in immunohistochemical studies as shown in Figure 5 (e) and Figure 5 (f).

The levels of total CREB protein and p-CREB were not significantly changed ($n = 6$, $p > 0.05$ vs. normal) in the CUS mice as shown in Figure 4(c) and Figure 4(d). Similarly, p-CREB/CREB ratio was not significantly decreased in the hippocampus of vehicle group ($n = 6$, $p > 0.05$ vs. normal) as shown in Figure 4(e), but it was significantly increased by fluoxetine ($n = 6$, $p < 0.05$ vs. vehicle) and LBP at the dose of 400 mg/kg ($n = 6$, $p < 0.05$, vs. vehicle). Significant increases in p-CREB level were seen ($n = 6$, $p < 0.05$, vs. vehicle) in the hippocampus of CUS mice as shown in Figure 4(d).

Analogous results from immunohistochemical studies are shown in Figure 5(f). The levels of p-CREB were significantly decreased by CUS in CA1, CA3 and DG ($n = 6$, $p < 0.05$, $p < 0.01$ vs. normal), but this effect was reversed by fluoxetine ($n = 6$, $p < 0.01$ vs. vehicle). LBP treatment at doses of 40 and 400 mg/kg significantly reversed-CREB expressions in CA1, CA3 and DG regions ($n = 6$, $p < 0.01$ vs. vehicle). On the other hand, LBP at the dose of 125 mg/kg significantly increased p-CREB level in CA1 region ($n = 6$, $p < 0.05$ vs. vehicle). Thus, LBP enhanced the levels of p-CREB in CUS mice. Since LBP also up-regulated BDNF levels, these results suggest that LBP can reverse CUS-induced depression-like behavior and cognitive deficits by upgrading CREB-BDNF signaling pathway in CUS mice.

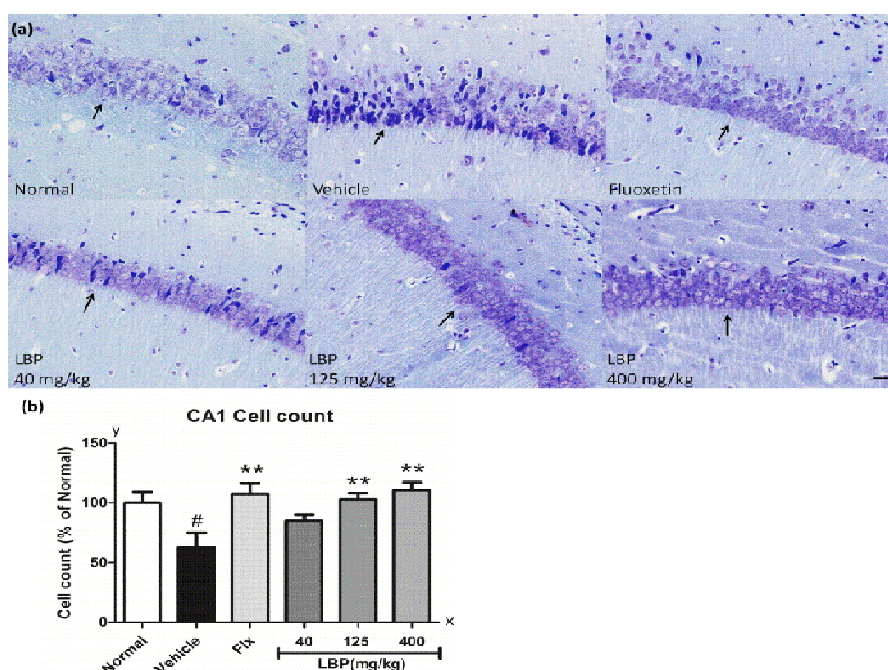


Figure 3: LBP treatment relieved CUS-induced histomorphology changes in the CA1 region of hippocampus by Nissl staining. **Note:** (a) The representative images of Nissl staining in the CA1 region of hippocampus. Note the neuronal cell shrinkage of the Vehicle group in CA1 region, as compare to the other groups, and as indicate by the black arrows. (b) LBP treatment relieves CUS-induced neuronal loss in CA1 region. The scale bar is 50 μ m for CA1 images. These data are represented as means + SEM ($n = 6$); ** $p < 0.01$, significantly different from vehicle; # $p < 0.05$, significantly different from normal; one-way ANOVA followed by Dunnett test

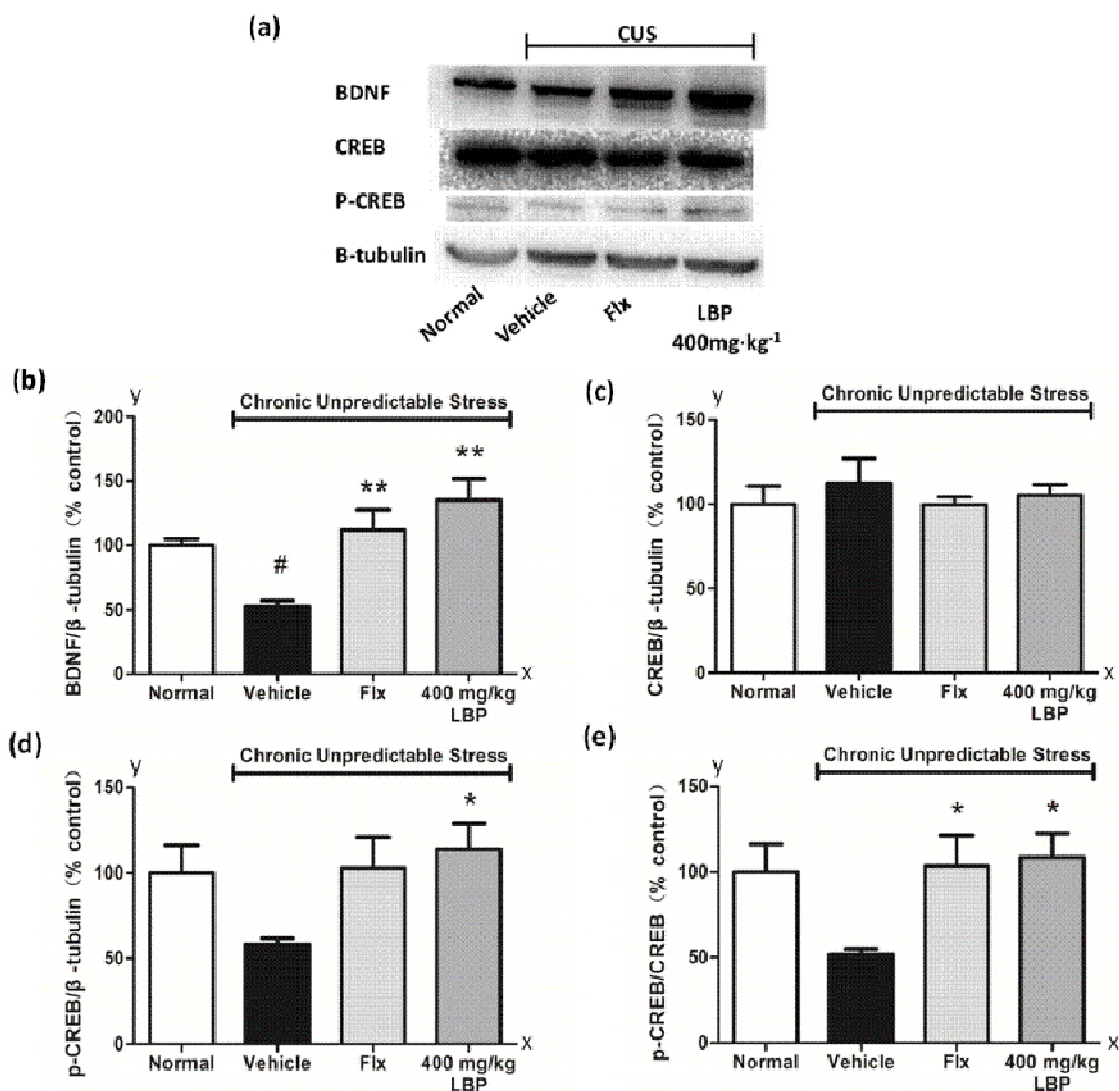


Figure 4: LBP treatment restores the CUS-induced decrease in the BDNF signaling pathway in the hippocampus by Western blotting. **Note:** (a) The Western blotting results. (b) Western blotting results showed that LBP treatment restores the CUS-induced decrease of BDNF in the hippocampus. (c) The changes of hippocampus CREB level between CUS and LBP mice were not significant. (d) LBP enhance the p-CREB level of CUS mice. (e) LBP can restore relative ratio of phosphorylation-CREB in hippocampus. These data are represented as means $\pm$ SEM ($n = 6$); * $p < 0.05$, ** $p < 0.01$, significantly different from Vehicle; # $p < 0.05$, significantly different from Normal; one-way ANOVA followed by Dunnett test

DISCUSSION

In the present study, it has been demonstrated that LBP can produce protective effects against chronic stress-induced cognitive dysfunction in mice models by modulating the CREB-BDNF signaling pathway in the hippocampus.

The use of complementary and alternative medicine by individuals with stress-induced psychopathological dysfunction has been on the increase [20,21]. Complementary medicine offers new hope to patients on account of its greater safety and fewer side-effects when compared to orthodox medicine.

Wolfberry (*Lyciumbarbarum* L., Solanaceae), as a type of functional food and folk medicine in China and East Asia, is now very popular in Western countries where it is marketed in form of health food products and anti-aging remedy. As a popular folk medicine in China, Wolfberry is used in traditional Chinese medicine [1]. LBP is an extract from *Lyciumbarbarum* fruits. It contains proteins, antioxidants, galacturonic acid, neutral sugars, arabinose, galactose, rhamnose, xylose, glucuronic acid, mannose and glucose [1].

The CUS model is the most adopted animal model for use in stress-related mental disorder research [6-8].

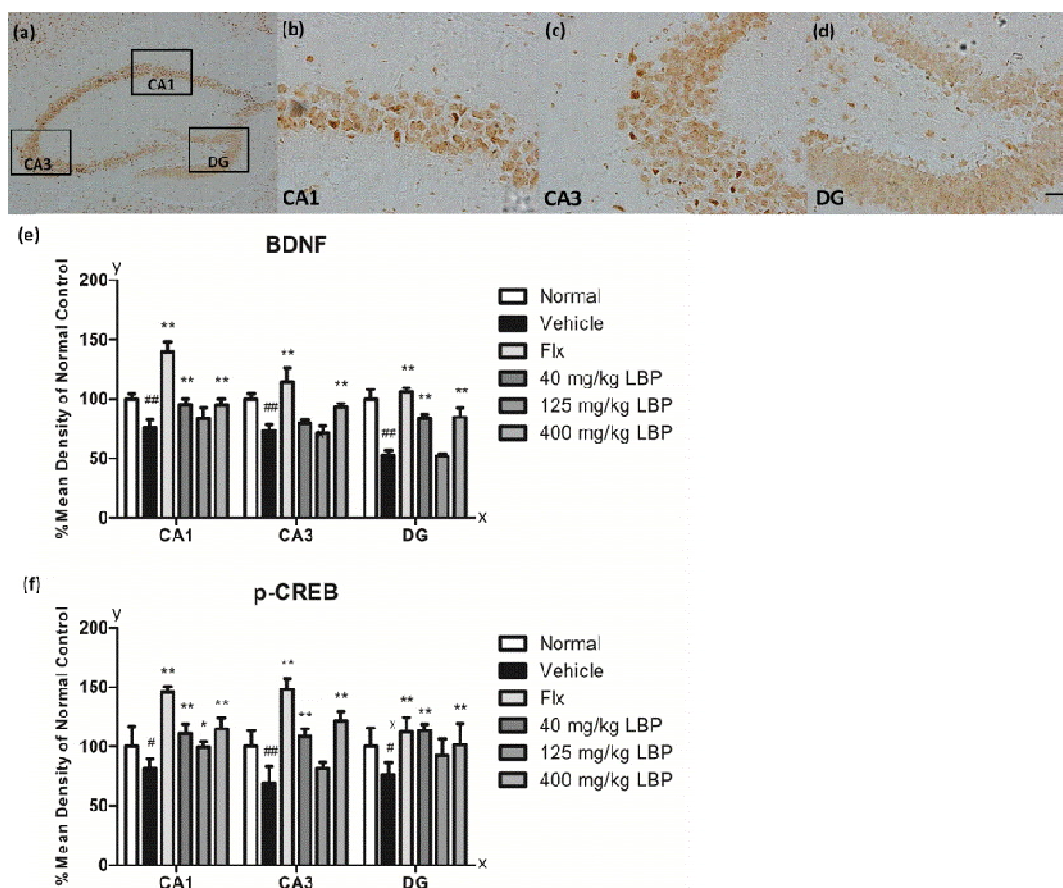


Figure 5: LBP treatment restores the CUS-induced deficit in the BDNF signaling pathway in the hippocampus as shown by immunohistochemical staining.

Note: (a) The synopsis immunohistochemistry picture of hippocampus. (b) – (d): The synopsis immunohistochemistry picture of CA1, CA3 and DG region. (e) LBP restores the CUS-induced decrease of BDNF in the hippocampus. (f) LBP restores the CUS-induced decrease of p-CREB in the hippocampus. The scale bar is 750 μ m for hippocampal images (a) and 75 μ m for CA1, CA3 and DG images (b - d) respectively. These data are represented as mean + SEM (n = 6); *p < 0.05, **p < 0.01, significantly different from vehicle; #p < 0.05, ##p < 0.01, significantly different from Normal; one-way ANOVA followed by Dunnett test

In an attempt to elucidate the effects of chronic LBP treatment on stressed animals, a 4-week design of stress procedure was employed to evaluate the results in this study [8], and the effectiveness of CUS procedure was monitored by mice performance in the OFT [17-19,22,23]. Changes in serum corticosterone levels were also measured in order to confirm the anticipated effect LBP treatment in CUS mice [24]. The data showed that chronic LBP treatment significantly improved mice performance in behavioral alterations induced by unpredictable stress stimulation. Moreover, LBP reversed the impaired feedback regulation of the HPA axis induced by CUS procedure.

Cognitive impairment is a core endophenotype of major depression [6,7]. Pretreatment with antidepressants can prevent the cognitive deficit caused by CUS [7,11]. These results show that chronic stress can influence the hippocampus in

a highly dynamic manner, such as spatial learning and memory. Chronic stress can produce neuronal loss and nuclear shrinkage in pyramidal neurons [11,25]. Studies have shown that these neuropathological deficits may be related to prolonged activation of the HPA axis and corticosteroid during stress, and can be reversed by antidepressant treatment [24].

In the present study, 4 weeks of CUS induced cognitive deficits in the performance of adult male ICR mice on MWMT was observed, in addition to remarkable neuronal morphological damage in CA1 region in hippocampus and elevation of plasma corticosterone levels. Chronic LBP treatment significantly relieved impairment of mice spatial learning and memory retention during CUS procedure, which is accompanied by relief of neuronal loss and nuclear shrinkage in pyramidal neurones in the CA1 region of the hippocampus.

From these results, it is evident that LBP ameliorated the behavioral alterations, such as cognitive dysfunction, in CUS-treated mice by attenuating plasma corticosterone levels and producing neuroprotective effects in the hippocampus.

BDNF plays an important role in morphological integrity of adult neurons and the activity of antidepressants in depressed patients, and in animal models of stress [9,11-14]. Various stress can decrease BDNF levels in the hippocampus, but the decrease can be reversed by antidepressant treatment. Thus, increases in BDNF expression might be a common mechanism of action of antidepressants against stress.

In this study, it was found that chronic LBP treatment can reverse the down-regulated expression of BDNF and ameliorate neuropathological deficits in hippocampus of the CUS mice. In order to further confirm the role of BDNF in the protective effect of LBP in CUS-treated mice, the effect of LBP on the transcription factor CREB activity was studied. CREB is an important regulator of BDNF-induced gene expression involved in BDNF signaling pathway [9]. The level of phosphorylated CREB is reduced in animal models of depression and depressed patients, and major classes of antidepressants can potentiate the level and/or function of phosphorylated CREB in several brain regions [9,11-14]. Enhancement of the CREB-BDNF signaling pathway could be a specific marker of restoration of stress-induced cognitive deficits. In this study, the results showed that chronic LBP treatment potentiated the expression of p-CREB in the hippocampus of stressed mice.

This study shows that LBP can produce beneficial effects against stress-induced cognitive dysfunction in mice models of stress. The effect appears to be mediated through the up-regulation of the CREB-BDNF signaling pathway in the hippocampus. This newly discovered effect of LBP provides a new insight for understanding the beneficial effects of LBP against stress-induced psychopathological dysfunction.

CONCLUSION

In this study, behavioral pharmacology, immunohistochemistry and biochemical assays have been employed to demonstrate the neuroprotective effects of LBP with respect to reversal of cognitive dysfunction induced by chronic unpredictable stress (CUS) in mice. The results

suggest that LBP exerts protective effects against cognitive dysfunction by reducing serum corticosterone levels and preventing neuronal morphological damage in the hippocampus, as well as up-regulating expression of CREB-BDNF signal pathway in the hippocampus during the CUS procedure. These results provide a new vista for exploiting *Lycium barbarum* as an alternative neuro-protective agent against stress-induced psychopathological dysfunction.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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