Antidepressant-like effect of Butea superba in mice exposed to chronic mild stress and its possible mechanism of action

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Abstract

Ethnopharmacological relevance: Butea superba (BS) is a Thai medicinal plant that has been used as a folk medicine to improve physical and mental conditions and to prevent impaired sexual performance in middle-aged or elderly males. We have previously reported that this plant extract could improve cognitive deficits and depression-like behavior in olfactory bulbectomized mice, an animal model of dementia and depression.

Aim of the study: In this study we examined the effect of BS on depression-like behavior in mice subjected to unpredictable chronic mild stress (UCMS) to clarify the antidepressant-like activity of BS and the molecular mechanism underlying this effect.

Materials and methods: UCMS mice were administered BS daily (300 mg of dried herb weight/kg, p.o.) or a reference drug, imipramine (IMP, 10 mg/kg, i.p.), 1 week after starting the UCMS procedure. We employed the sucrose preference test and the tail suspension test to analyze anhedonia and depression-like behavior of mice, respectively. Serum and brain tissues of mice were used for neurochemical and immunohistochemical studies. The UCMS procedure induced anhedonia and depression-like behavior, and BS treatment, as well as IMP treatment, attenuated these symptoms. UCMS caused an elevation of serum corticosterone level, an index of hyper-activation of the hypothalamic–pituitary–adrenal (HPA) axis, in a manner attenuated by BS and IMP treatment. BS treatment also attenuated UCMS-induced decrease in the expression levels of brain-derived neurotrophic factor (BDNF) mRNA, cyclic AMP-responsiv element binding protein (CREB) and a phosphorylated form of N-methyl-D-aspartate receptor subunit NR1, synaptic plasticity-related signaling proteins. Moreover, the UCMS procedure reduced doublecortin-positive cells in the dentate gyrus region of the hippocampus. BS administration reversed these UCMS-induced neurochemical and histological abnormalities.

Conclusion: These results suggest that BS can ameliorate chronic stress-induced depression-like symptoms and that the effects of BS are mediated by restoring dysfunctions of the HPA axis and synaptic plasticity-related signaling systems and neurogenesis in the hippocampus.

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1. Introduction

Depression is a common psychiatric disorder with a lifetime prevalence of 12–17% of the population worldwide (Kessler et al., 2003). This disorder is characterized by psychological, behavioral and physiological symptoms such as anhedonia, low mood, loss of interest, etc. and arises from the interaction between genes and environmental factors including stress (Uher, 2008; Dalla et al., 2010; Zhu et al., 2012; Kessler and Bromet, 2013). Although various antidepressant drugs have been clinically used for the treatment of patients with depression, most of the patients recover incompletely, have recurrent episodes, or suffer from many serious adverse effects. Moreover, the molecular mechanisms behind depressive symptoms are still far from clearly understood. Thus, further investigation and establishment of new therapeutic methods/drugs with an efficient anti-depressant activity and fewer side effects are greatly needed.

Butea superba (BS) (Red Kwao Kua in Thai) is an herb in the family Papilionaceae (Leguminosae), which is abundantly distributed in Thai
deciduous forests. The tuberous roots of this plant have been used for centuries as a folk medicine not only to improve physical and mental conditions but also to prevent impaired sexual performance in middle-aged or elderly males (Suntara, 1931; Tocharus et al., 2006). Clinical studies demonstrated that oral administration of powdered tubers of this plant elicited noteworthy amelioration in middle-aged males with erectile dysfunction, which might be related to the fact that BS has some isoflavonolignans such as butespuberin A and B, which have inhibitory activities against phosphodiesterase type 3A and type 5, enzymes targeted for erectile dysfunction therapy (Cherdshewasart and Nimsakul, 2003; Ma et al., 2005; Tocharus et al., 2006). In addition, our previous study using olfactory bulbectomized (OBX) mice as an animal model of dementia and depression clarified that this plant can ameliorate cognitive dysfunction of OBX animals via normalizing synaptic plasticity-related signaling and facilitating central cholinergic systems (Mizuki et al., 2014). We also found that BS attenuates depression-like behavior via a mechanism differing from that implicated in BS amelioration of their cognitive function in OBX animals (Mizuki et al., 2014), leading to the hypothesis that BS has a potential application to depression, too. However, this hypothesis remains to be tested in detail using an animal model of depression with more appropriate validity.

The unpredictable chronic mild stress (UCMS) model is an animal model of depression that has a combination of predictive validity, face validity and construct validity (Duman, 2010; Willner, 2005). Indeed, the UCMS procedure was shown to induce pathophysiological alterations relevant to depression, such as hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis, anhedonia, down-regulation of brain-derived neurotrophic factor (BDNF), cyclic AMP-responsive element-binding protein (CREB) and neurogenesis in the hippocampus, and these impairments could be reversed by antidepressant drugs (Ito et al., 2006; Kuipers et al., 2013).

In the present study, we aimed to clarify the antidepressant drug-like effects of BS using a mouse model of UCMS and to reveal a plausible mechanism underlying these effects. Our findings demonstrated that BS attenuates depression-like behavior of UCMS animals and that these effects are associated with normalization of CMS-induced deterioration of BDNF-CREB signaling, abnormalities of the HPA axis and the suppression of neurogenesis, indicating that BS extract is beneficial for the treatment of stress-induced depressive disorder.

2. Materials and methods

2.1. Animals

This study was conducted according to the experimental protocols described in Fig. 1. A total of 72 male ddY mice (Japan SLC Inc., Shizuoka, Japan) were obtained at the age of 7 weeks old. The animals were habituated to the laboratory animal room for 1 week before UCMS treatment. Food and water were available ad libitum. Housing was thermostatically maintained at 24 ± 1 °C with constant humidity (65%) and a 12-h light–dark cycle (lights on: 07:00–19:00). The behavioral experiments were performed during the light phase from 9:00 to 18:00. The present studies were conducted in accordance with the Guiding Principles (NIH publication #BS-23, revised in 1985) for the Care and Use of Animals and were approved by the Institutional Animal Use and Care Committee of the University of Toyama.

2.2. Preparation of plant extract

The ethanol extract of Butea superba (BS) was prepared as previously described. Briefly, the root of BS was collected in Lampang Province in Thailand in 2011 and identified by Dr. Chaipy Chaichantipyuth (Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University). The dried powder of the root (52 g) was extracted with 200 ml of 95% ethanol at 75 °C for 2 h and filtrated. This step was repeated 3 times and the filtrated samples were combined, concentrated under reduced pressure at 40 °C and then dried in vacuo. The yield of the extraction from the dried root was calculated as 8.9% (w/w). The BS (voucher specimen no. TMPW-27997) and its extract (voucher specimen no. INM-531) used in this study were deposited at our institute.

To identify the chemical constituents of BS, liquid column chromatography–mass spectrometry (LC–MS) analyses were performed with a Shimadzu LC–IT–TOF mass spectrometer equipped with an ESI interface (Shimadzu, Kyoto) as previously described (Yamada et al., 2011; Le et al., 2013; Mizuki et al., 2014). The ESI parameters were as follows: source voltage –4.5 kV, capillary temperature 200 °C and nebulizer gas 1.5 l/min. The mass spectrometer was operated in positive ion mode scanning from m/z 200 to 2000. A Waters Atlantis T3 column (2.1 mm i.d. × 150 mm) was used and the column temperature was maintained at 40 °C. The mobile phase was a binary eluent of (A) 5 mM ammonium acetate solution and (B) acetonitrile, under the following gradient conditions: 0–30 min linear gradient from 10% to 100% B and 30–40 min isocratic at 100% B. The flow rate was 0.2 ml/min. Mass spectrometry data obtained from the extract have been listed in the MassBank database (Horai et al., 2010) and stored together with the pharmacological information on the extract in the Wakan-Yaku Database system (http://wakandb.u-toyama.ac.jp/wiki/LCMS:Butea_INM-531), Institute of Natural Medicine, University of Toyama.

2.3. Unpredictable chronic mild stress procedure

The animals were randomly divided into 5 groups: a non-stressed control group and 4 other groups subjected to UCMS for 6 weeks. The UCMS procedure (Monleón et al., 1995; Song et al., 2006) was used with minor modification. The procedure consisted of a variety of unpredictable mild stressors including one 18-h period of food and water deprivation before the measurement of 10% sucrose consumption, two periods of cage tilting at 45° (12 h), two periods of restricted access to food (1 h), two periods of exposure to an empty bottle (3 h), one 21-h period of a wet cage (200 ml of water in 50 g of sawdust bedding), two periods of light exposure (36 h), three periods of intermittent sound (3 h) and two periods of paired caging (2 h). These stressors were randomly scheduled over a one-week period and repeated throughout the 6-week experiment. The non-stress control group was housed under normal conditions.

![Fig. 1. Schematic drawing of the experimental protocol. Week 7-week-old ddY mice were divided into a non-stress group and an unpredictable chronic mild stress (UCMS) group at week 0. The UCMS group received various unpredictable stressful stimuli from week 0 to week 7. The UCMS group was administered vehicle tap water or test drugs daily (Butesuperba extract: 300 mg/kg per day, p.o.; imipramine: 10 mg/kg per day, i.p.) from week 1 to week 7. The UCMS exposure period from week 0 to week 6, sucrose preference test and tail suspension test were conducted according to the methods described in the text. One week after the tail suspension test, the animals were decapitated and the brain tissues were collected for neurochemical experiments.](image-url)
2.4. Drug administration

Except in specially stated cases, either vehicle water or test drugs were administered daily after a 1-week habituation period. On a behavioral testing day, administration was conducted 1 h before the testing. The vehicle-treated non-stress mice were per orally administered tap water. The reference standard drug, imipramine HCl (IMP), was dissolved in 0.9% saline and administered once daily at a dose of 10 mg/kg (i.p.). BS extract was dissolved in tap water and given orally at daily doses of 300 mg (dried herb weight)/kg. We used this dose of BS because, in our previous study where the effect of BS (100–300 mg/kg per day, p.o.) was examined using a mouse model of olfactory bulbectomy, it exhibited a significant anti-depressant-like profile at 300 mg/kg per day.

2.5. Behavioral studies

2.5.1. Sucrose preference test

The sucrose preference test was conducted once a week (a total of 6 times) after the end of the UCMS. Before the test, the animals were housed individually in a cage and deprived of food and water for 18 h. In this test, two drinking nozzles were set at the cage through which the animal could intake tap water or a 10% sucrose solution for 1 h (Berry et al., 2012; Pothion et al., 2004). In each test, the position of the nozzles was switched to counterbalance the effect of position preference according to previous reports (Kant and Bauman, 1993; Strekalova et al., 2004). The amounts of water and sucrose solution consumed during a 1-hr period were recorded. Sucrose preference of each animal was calculated as follows:

Sucrose preference (%) = S/(S+W)100.

Here, S and W represent the amounts of 10% sucrose solution and water consumed (ml), respectively.

2.5.2. Tail suspension test

We employed a tail suspension test to assess the antidepressant effects of the test drugs (Shioda et al., 2010). This test was conducted as previously described (Sithisarn et al., 2013; Mizuki et al., 2014). Briefly, each mouse was individually suspended 50 cm above the floor by the tail with adhesive tape placed approximately 2 cm from the tip of the tail. This short-term inescapable stress led to development of an immobile posture. The animal behavior in the test was video-recorded for later analysis. Immobility was defined as a state with movement speed of no more than 0.05 cm²/s using the SMART® system ver. 2.5 and was recorded for 8 min. The performance during the last 6-min period was analyzed.

2.6. Neurochemical studies

2.6.1. Serum corticosterone level

Blood samples were collected immediately after decapitation and centrifuged at 3000 rpm and 4 °C for 15 min to isolate serum. Serum was stored at −20 °C until use. The serum level of corticosterone was determined using an AssayMax Corticosterone ELISA kit (Assaypro LLC, St. Charles, MO) according to the manufacturer’s instructions. The minimum detectable dose of corticosterone is typically ~0.3 ng/ml. Intra-assay and inter-assay coefficients of variation were 5.0% and 7.2%, respectively.

2.6.2. Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)

Q-PCR was conducted as previously described (Zhao et al., 2007; Zhao et al., 2011; Le et al., 2013). Total RNA was extracted from the hippocampus with Sepazol® (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s instructions. First-strand cDNA was synthesized with oligo(dT) primers and M-MLV reverse transcriptase® (Invitrogen, Rockville, MD, USA) and used as a template for real-time PCR. Q-PCR was carried out with Fast SYBR Green Master Mix and the StepOne Real-time PCR System® (Applied BioSystem, USA). The following primers were synthesized by Nippon EGT Co. (Toyama, Japan): BDNF (NM_007540), 5′-AGCTGACGCTGTGACAGT-3′ (forward) and 5′-TCCATATAGCCCGAAC-3′ (reverse); β-actin (NM_007393): 5′-CATGCTTAAGAGCCCTTACTGACCG-3′ (forward) and 5′-ATGGAGCCACCGATCCACA-3′ (reverse); neurogenin 2 (Ngn2) (AC_0000251): 5′-AATCCTACCCCTTACAG-3′ (forward) and 5′-GGAGGCAGTAACGTCCT-3 (reverse). Melting curve analysis of each gene was performed each time after amplification. In all reactions, β-actin mRNA was used as a control to which the results were normalized. Standard curves of the log concentration of each gene vs. cycle threshold were plotted to prove inverse linear correlations. The correlation coefficients for standard curves of target genes were 0.889–1.0.

2.6.3. Western Blotting

The expression of synaptic plasticity-related proteins in the hippocampus was analyzed using Western blotting as previously described (Inada et al., 2013; Le et al., 2013; Mizuki et al., 2014). The following primary antibodies were used: anti-NMDAR1 rabbit polyclonal antibody (1:1000 dilution), anti-phospho-NMDAR1 (p-NMDAR1) (pSer896) rabbit polyclonal antibody (1:1000 dilution) (Cell Signaling Technology, USA), anti-glutamate receptor 1 (Glur1) rabbit polyclonal antibody (1:1000 dilution), anti-phospho-GluR1 (p-GluR1) (pSer 831) rabbit polyclonal antibody (1:1000 dilution) (Sigma-Aldrich, Co., St. Louis, USA), anti-CalM/Kl (A-1: sc-13141) mouse monoclonal antibody (1:1000 dilution), anti-phospho-CalM/KII (p-CalM/KII) (pThr286) rabbit polyclonal antibody (1:1000 dilution), anti-CREB (48H2) rabbit monoclonal antibody (1:1000 dilution), anti-phospho-CREB (p-CREB) (pSer133) rabbit monoclonal antibody (1:1000 dilution) and anti-β-actin mouse monoclonal antibody (1:10,000 dilution, Abcam®, Cambridge, UK). The immunocomplexes were detected by the enhanced chemiluminescence method (ImmobilonTM Western Chemiluminescent HRP Substrate) (Millipore, Temecula, CA, USA) and imaged using Lumino Image Analyzer LAS-4000R (Fuji Film, Tokyo, Japan). The quantity of immune-reactive bands was analyzed using ImageQuant TL software (GE Healthcare, Buckinghamshire, UK). The quantity of immunoreactive bands was normalized by comparison with their expression levels in treatment-naïve control mice. The expression levels of each target protein were re-probed using a Blot Restore Membrane Rejuvenation Kit (Millipore, Temecula, CA, USA).

2.7. Immunostaining of a neurogenesis marker in the dentate gyrus

Mice were fixed by intracardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS) under pentobarbitral anesthesia. Brains were post-fixed with 4% paraformaldehyde PBS for 24 h and cryo-protected in 30% sucrose/PBS for an additional 24 h at 4 °C. Serial coronal brain sections (20 μm) were cut on a cryostat (Leica CM3050, Germany) and kept at −20 °C until use. Newly generated neurons in the dentate gyrus were elucidated by staining Dcx-immunopositive cells. After gentle washing with PBS, sections were incubated in Target Retrieval Solution (Dako, Japan) for 30 min at 98 °C to activate antigen. The slice sections were blocked with 10% normal goat serum in PBS for 1 h at room temperature and then incubated with anti-Dcx antibody (rabbit polyclonal antibody 1:500 dilution, ab18723; Abcam, Cambridge, UK) at 4 °C overnight. After washing with PBS, the sections were incubated in goat anti-rabbit IgG antibody (1:300; Alexa Fluor 594,
A11037, Invitrogen, Tokyo, Japan) for 1 h and then mounted with DAPI-containing medium. Images were captured using an upright optical microscope under a 20 × objective (BX-61, Olympus, Tokyo, Japan) equipped with a digital camera (DP50, Olympus, Tokyo, Japan). Dcx-positive cells were counted in the dentate gyrus area (400 × 500 μm²) of brain sections at −2.06 mm relative to the bregma (2–3 sections of each animal) in a blind manner.

2.8. Data analysis

The data are expressed as the mean ± SEM. The data obtained from the behavioral tests and neurochemical experiments were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls test for multiple comparisons, and the sucrose preference test was analyzed by two-way repeated measures ANOVA (one factor repetition). Differences of p < 0.05 were considered significant. The analysis was conducted using SigmaStat® ver. 3.5 (SYSTAT Software Inc., Richmond, CA, USA).

3. Results

3.1. Effects of BS and IMP on UCMS-induced depressive-like symptoms in the sucrose preference test and the tail suspension test

First, we verified whether our chronic mild stress procedure can induce depressive-like symptoms in mice. In the sucrose test, we aimed to examine UCMS-induced anhedonia. As shown in Fig. 2, sucrose intake of the vehicle-treated UCMS group was significantly less preference for sucrose solution than the non-stress mice [F(2,3)=13.612, p = 0.005; F(5,22)=2.405, p = 0.078; F(5,22)=3.077, p = 0.012] in weeks 4 (p < 0.001) and 6 (p = 0.004). BS administration significantly restored sucrose preference decreased by UCMS to the level of the non-stress control animals [F(2,3)=13.222, p = 0.005; F(5,22)=2.349, p = 0.046; F(5,22)=4.256, p = 0.001]. In fact, sucrose intakes of the BS-treated UCMS group in weeks 4 (p < 0.001), 5 (p = 0.021) and 6 (p < 0.001) were significantly greater than those of the vehicle-treated UCMS group.

In the tail suspension test, the vehicle-treated UCMS mice showed significantly prolonged immobility time compared with the non-stress control mice, and BS treatment significantly shortened the immobility time of UCMS animals like IMP treatment did [one-way ANOVA: F(2,3)=6.070, p = 0.006; post hoc test: non-stress group vs. vehicle-treated UCMS group, p = 0.005; vehicle-treated UCMS group vs. BS-treated UCMS group, p = 0.013].

3.2. BS reduces serum corticosterone level in UCMS mice

We analyzed the serum level of corticosterone to clarify whether the feedback mechanism in the HPA axis was impaired by exposure to our UCMS procedure (Fig. 4). Compared with the non-stress control mice, the vehicle-treated UCMS mice had a significantly increased level of serum corticosterone. However, daily treatment with BS (300 mg/kg per day, p.o.) and IMP (10 mg/kg per day, i.p.) suppressed UCMS-induced elevation of the corticosterone level [one-way ANOVA: F(3,8)=11.162, p = 0.003; post hoc test: non-stress group vs. vehicle-treated UCMS group, p = 0.003; vehicle-treated UCMS group vs. BS-treated UCMS group, p = 0.005; vehicle-treated UCMS group vs. IMP-treated UCMS group, p = 0.003; BS-treated UCMS group vs. IMP-treated UCMS group, p = 0.883].

![Fig. 2. The effects of daily administered imipramine and Butea superba extract on UCMS-induced decrease in sucrose preference in mice. The amount of 10% sucrose solution taken by each animal group was measured as an index of sucrose preference once a week as stated in the text. Imipramine (IMP: 10 mg/kg per day, i.p.) and Butea superba extract (BS: 300 mg/kg per day, p.o.) were daily administered during the experimental period. Each data point represents the mean ± S.E.M. (n = 12 in each animal group). *p < 0.05 vs. vehicle-treated non-stress group. #p < 0.05 and ##p < 0.01 vs. respective vehicle-treated CMS group [two-way repeated measures ANOVA (one factor repetition)].](image-url)
Fig. 3. The effects of daily administration of imipramine and Butea superba extract on UCMS-induced depression-like behavior in the tail suspension test. Immobility of each animal group was measured as an index of depression-like behavior 6 weeks after starting UCMS treatment. Animal behavior in tail suspension was video-recorded and immobility was analyzed using the SMART system as described in the text. Each data column represents the mean ± S.E.M. (n = 12 in each animal group). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. vehicle-treated UCMS group (ANOVA followed by post hoc Student–Newman–Keuls test).

Fig. 4. The effects of imipramine and Butea superba extract on UCMS-induced increase in serum corticosterone level. One week after completing the behavioral studies, the animals were decapitated to collect blood samples as well as brain tissues. The corticosterone level was measured as detailed in the text. Each data column represents the mean ± S.E.M. (n = 3 in each animal group). *p < 0.05 and **p < 0.01 vs. vehicle-treated UCMS group (ANOVA followed by post hoc Student–Newman–Keuls test).

3.3. BS ameliorates UCMS-induced dysfunction of NMDAR1 and BDNF-CREB systems

We next examined the expression level of BDNF gene transcript, which plays a pivotal role in neuronal survival and synaptic plasticity (Fig. 5). The vehicle-treated UCMS mice had a significantly reduced level of BDNF mRNA in the hippocampus. On the other hand, BS (300 mg/kg/day) and IMP-treated UCMS mice significantly reversed the decrease in the expression level of BDNF mRNA [one-way ANOVA: F(3,16) = 12.921, p < 0.001; post hoc test: non-stress group vs. vehicle-treated UCMS group, p = 0.001; vehicle-treated UCMS group vs. BS-treated UCMS group, p = 0.001; vehicle-treated UCMS group vs. IMP-treated UCMS group, p = 0.001]. We further analyzed the effect of BS treatment on signaling proteins implicated in synaptic plasticity in the hippocampus (Fig. 6). No differences in the expression levels of NMDAR1 (NMDAR subunit), GluR1 (AMPAR subunit) or CREB were found among the vehicle-, BS- and IMP-treated UCMS groups. However, compared with the non-stress control group, the vehicle-treated UCMS group showed a significant decrease in the levels of pSer896-NMDAR1 [one-way ANOVA: F(3,12) = 5.450, p = 0.013] and pSer133-CREB [one-way ANOVA: F(3,12) = 7.091, p = 0.005] vs. vehicle-treated UCMS group, p = 0.004]. BS and IMP reversed the UCMS-induced down-regulation in pSer896-NMDAR1 [post hoc test: vehicle-treated UCMS group vs. BS-treated UCMS group, p = 0.020; vehicle-treated UCMS group vs. IMP-treated UCMS group, p = 0.012] and pSer133-CREB [post hoc test: vehicle-treated UCMS group vs. BS-treated UCMS group, p = 0.009; vehicle-treated UCMS group vs. IMP-treated UCMS group, p = 0.018]. On the other hand, the expression level of pSer831-GluR1 in the hippocampus was not significantly affected by ether stress exposure or treatment with BS and IMP [one-way ANOVA: F(3,12) = 2.387, p = 0.120]. Moreover, there were no significant differences in the expression levels of pSer896-NMDAR1 [post hoc test: BS-treated UCMS group vs. IMP-treated UCMS group, p = 0.926] and pSer133-CREB [post hoc test: BS-treated UCMS group vs. IMP-treated UCMS group, p = 0.627] between BS-treated UCMS group and IMP-treated UCMS group.

3.4. BS attenuates UCMS-induced suppression of neurogenesis in the hippocampus

Since alteration of neurogenesis in the dentate gyrus region of the hippocampus is one of the indices relevant to chronic stress...
and is susceptible to anti-depressant treatment, we elucidated the
effect of repeated BS treatment on the number of cells stained by
Dcx, a marker of neurogenesis and immature neurons, in the
dentate gyrus of UCMS animals. As shown in Fig. 7, the number of
Dcx-positive cells was significantly decreased in the vehicle-
treated UCMS group compared with that in the non-stress control
group [one-way ANOVA: F(3,9) = 12.572, p = 0.001; post hoc test:
vehicle-treated UCMS group vs. non-stress group, p = 0.006]. On
the other hand, the UCMS-induced decreases in Dcx-positive cells
were significantly reversed in the UCMS groups treated daily with
BS as well as with IMP [post hoc test: vehicle-treated UCMS vs. BS-
treated UCMS group, p = 0.003; vehicle-treated UCMS group vs.
IMP-treated UCMS group, p = 0.001]. No significant difference in
the number of Dcx-positive cells was found between BS-treated
and IMP-treated UCMS groups (p = 0.284).

3.5. Ngn2 mRNA expression is not related to neurogenesis
in the BS-treated mice

To investigate further whether the effect of BS extract on
neurogenesis in the dentate gyrus of UCMS animals is due to
facilitation of proliferation and differentiation of neural stem cells,
we analyzed the expression level of the gene encoding neuro-
genin2 (Ngn2), a factor implicated in the differentiation of neural
stem cells into neurons, in the hippocampus, since BS reportedly
activates the Ngn2 promoter in C17.2 neural stem cells (Arai et al.,
2013). In the present study, quantitative real-time PCR analysis
revealed that the UCMS procedure had no effect on the expression
level of Ngn2 mRNA in the hippocampus [non-stress group: 0.271 ± 0.017, vehicle-treated UCMS group: 0.309 ± 0.016; BS-
treated UCMS group: 0.291 ± 0.006, and IMP-treated UCMS group:
0.307 ± 0.024 (mean ± S.E.M. of Ngn2 mRNA/β-actin mRNA ratio,
n = 4)]. Neither BS nor IMP treatment altered the hippocampal
expression level of Ngn2 mRNA in the UCMS groups [one-way
ANOVA: F(3,12) = 1.210, p = 0.348].

4. Discussion

This study aimed to determine the anti-depressant effect of BS
extract and its mechanism(s) of action using UCMS animals as an
animal model of depression. The present findings demonstrated
that BS ameliorates depression-like behavior of UCMS mice via
reversing dysfunction of the synaptic plasticity-related neuro-
signaling system and neurogenesis in the hippocampus and
over-activation of the HPA axis in UCMS animals.

We first analyzed the effects of BS on UCMS-induced anhe-
donia-like behavior (Taksande et al., 2013) and depression-like
behavior using the sucrose preference test and tail-suspension
test, respectively. Evidence indicates that anhedonia is one of the
core symptoms of patients with major depression and is respon-
sive to anti-depressant treatment and that, in experimental
animals, this kind of symptom can be induced by the UCMS
paradigm as a reduction of preference for sweetened solutions.
It is of interest that repeated administration of BS, as well as of IMP, ameliorated UCMS-induced decrease in sucrose intake in the sucrose preference test and increase in immobility in the tail suspension test. In our previous study using a mouse model of olfactory bulbectomy, we suggested that BS possesses not only an anti-dementia drug-like effect but also anti-depressant-like activity (Mizuki et al., 2014). Taken together, the present results provide further evidence for the anti-depressant-like effect of BS and support the idea that BS administration may be beneficial for the treatment of dementia patients with depressive symptoms (Mizuki et al., 2014).

To obtain a better understanding of the mechanism(s) whereby BS administration shows an anti-depressant-like effect in UCMS animals, we first analyzed the serum corticosterone level as a biomarker of excess activation of the HPA axis (Shansky and Lipps, 2013) since it plays an important role not only in therapeutic mechanisms of antidepressant drugs (Kim et al., 2013) but also in the pathogenesis of endogenous depression (Ito et al., 2006). In this study, the animals exhibiting depression-like symptoms under the UCMS procedure had a significantly elevated level of serum corticosterone compared with the non-stress control animals and this elevation could be reversed by BS as well as by IMP. These results raise the possibility that repeated BS administration can

![Image of DCX-positive cells in the dentate gyrus](https://example.com/image1.png)

**Fig. 7.** The effects of imipramine and *Butea superba* extract on neurogenesis in the hippocampal dentate gyrus region of UCMS group newly generated neuronal cells in the dentate gyrus were immunohistochemically stained using anti-doublecortin (Dcx) antibody as a neurogenesis marker. (A) Typical photos presenting the immunostaining of doublecortin (DCX)-positive cells in the dentate gyrus. Arrows indicate Dcx-immunopositive cells. (B) Quantitative comparisons of the number of DCX-positive cells among different treatment groups. The number of immunopositive cells was counted in a blind manner. Each data column represents the mean ± S.E.M. (n=3–5 in each animal group). **p < 0.01 vs. vehicle-treated UCMS group (ANOVA followed by post hoc Student–Newman–Keuls test).
suppress UCMS-induced excess activation of the HPA axis and thereby attenuate depression-like symptoms of the UCMS animals.

This study demonstrated that, consistent with previous reports (Li et al., 2007; Kuijpers et al., 2013), the present UCMS procedure down-regulated BDNF mRNA expression and CREB phosphorylation in the hippocampus and that BS administration significantly reversed the effects of UCMS, as did IMP. Lines of evidence indicate that the CREB–BDNF signaling system can be affected by stress and is a potential target of antidepressant treatment. In fact, BDNF promotes neuronal survival, differentiation, function and plasticity and plays a key role in the pathophysiology of depression (Castren and Rantamaki, 2010; Li et al., 2013). In addition, the expression of BDNF mRNA can be regulated via a variety of signaling cascades converging into CREB, which is activated by phosphorylation of Ser133 (Tardito et al., 2006) and involved in the long-term effects of antidepressant via regulation of the expression of genes encoding the proteins implicated in synaptic plasticity (Nair and Vaidya, 2006; Freitas et al., 2013; Gundersen et al., 2013). Taken together, our findings suggest that repeated BS administration ameliorates UCMS-induced depression-like symptoms by normalizing the UCMS-induced dysfunction of BDNF-CREB signaling systems in the hippocampus.

Moreover, it is of interest that the present UCMS procedure also reduced the levels of phosphorylated forms of NMDA receptors (p-NR1) and AMPA receptors (Ser831) in the hippocampus, although the change in the p-GluR1 level was insignificant, and that the decrease in p-NR1 level was reversed by daily administration of BS and IMP. The effects of BS on the reduced levels of p-NR1 and p-CREB in UCMS mice agree with our previous study demonstrating that the down-regulated expression levels of these phosphorylated proteins in an OBX model of dementia and depression were reversed by BS administration (Mizuki et al., 2014). Evidence indicates that glutamate receptor-mediated neurotransmission is located upstream of the CREB–BDNF signaling systems (Zhao et al., 2009) and is a molecular basis underlying cognitive function (Lau et al., 2004). Stimulation of glutamate receptors can elicit phosphorylation of subunits (NR1–S896) of NMDAR and AMPAR at glutamatergic synapses, trigger CREB–BDNF signaling (Hardingham et al., 2002; Chen et al., 2008) and positively regulate each of receptor function, synaptic strength and synaptic plasticity (Lee, 2006; Chen and Roche, 2007). Therefore, taken together, our findings suggest that the decrease of glutamate receptor function observed in the vehicle-treated UCMS group is at least partly involved in the UCMS-induced behavioral alterations and that glutamatergic neurotransmission plays a role in the antidepressive effects of IMP and BS in UCMS animals.

In this study, we found that phosphorylated forms of GluR1 and CaMKII (Ser831) had less susceptibility to the UCMS procedure and IMP and BS treatment than NR1 and CREB. The exact reason for this difference is unclear, but one may infer that, in particular, phosphorylation of CaMKII is resistant to IMP and BS treatment in a UCMS model of depression since CaMKII is an enzyme implicated in the phosphorylation of GluR1 at Ser831. This possibility, however, can be ruled out because, in our previous study, OBX-induced decrease in the levels of p-CaMII and p-GluR1 (Ser831) in the hippocampus was reversed by repeated treatment with IMP and BS.

The present results regarding glutamate receptor function of UCMS animals differ from the reports from other laboratories. Calabrese et al. (2012) and Garcia et al. (2009) showed that UCMS induces hyperactivity of glutamatergic function in rat and that it was attenuated by the NMDA antagonist ketamine (Garcia et al., 2009). The reason for the conflict between the present and previous findings is unclear, but it may be due to differences in the species of animals and the procedure of UCMS employed.

One of the important findings in this study is that BS treatment as well as imipramine significantly reversed suppression of hippocampal neurogenesis by the UCMS procedure. Several lines of evidence indicate that the subgranular zone of the hippocampal dentate gyrus is one of the brain regions with continuous generation of neurons and that neurogenesis in the dentate gyrus plays an important role in various physiological functions such as learning and memory (Bram et al., 2007) and emotional behavior (Gould et al., 1997). Hippocampal neurogenesis is also susceptible to various stressful experiences, including UCMS, and can be stimulated by antidepressant drug treatment (Malberg et al., 2000). In this study, we found that BS treatment increased DCX-positive cells, which are marker cells representing neurogenesis and immature neurons, in the hippocampal subgranular zone in the UCMS group. Our findings raise the possibility that BS treatment facilitates both the proliferation of neural progenitor cells and the development of immature neuronal cells, like antidepressant drugs.

A recent in vitro study reported by Arai et al. (2013) demonstrated that some chemical constituents isolated from BS extract could activate Ngn2 promoter, which is reportedly expressed in adult hippocampal neural progenitor cells (Hodge et al., 2008) and facilitates adult neurogenesis by activating neuronal progenitors in the dentate gyrus (Ozen et al., 2007). These reports allow us to speculate that activation of Ngn2 promoter in the hippocampal dentate gyrus neuronal progenitor cells may be involved in the ameliorative effects of BS on UCMS-induced depression-like symptoms and the suppression of hippocampal neurogenesis. However, this seems very unlikely because the expression level of hippocampal Ngn2 mRNA was insusceptible to the UCMS paradigm or BS administration. Therefore, further investigation is required to clarify the mechanisms underlying BS–induced restoration of hippocampal neurogenesis suppressed in UCMS animals.

The present study showed no significant difference in the amelioration UCMS-induced behavioral and neurochemical changes between BS- and IMP-treated groups. These results allow us to consider the possibility that BS shares the same molecular mechanism with IMP to exhibit the anti-depressive effect in UCMS animals. Nevertheless, further studies are needed to clarify this possibility.

5. Conclusion

The present results suggested that BS ameliorates chronic stress-induced depression-like symptoms via restoring dysfunctions of the HPA axis and synaptic plasticity-related signaling systems and neurogenesis in the hippocampus. Considering the fact that BS has been used for long time as a folk medicine, it is likely that BS can be used to prevent/ameliorate depression-related symptoms attributable to chronic stress.

Authors’ contributions

HF and KM were responsible for the study concept and design. DM contributed to behavioral and neurochemical experiments. DM and KT conducted acquisition and analysis of chemical profiling data of BS extract. XTL supported immunohistochemical analysis of neurogenesis. YH and TI assisted with chemical data analysis and interpretation of findings. DM and KM drafted the manuscript. All authors read and approved the final version of the manuscript.


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