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Original Article

Protective effect of betulinic acid for treating unpredictable chronic mild stress-induced depression in mice by inhibiting brain RIP140 activation

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Abstract: The present study was designed to evaluate whether betulinic acid (BA) could exert an antidepressant-like effect in mice exposed to unpredictable chronic mild stress (UCMS) and to explain its underlying mechanisms. Behavioral changes, which was investigated through sucrose preference test (SPT), open field test (OFT), forced swimming test (FST) and tail suspension test (TST), indicated that BA (20 mg/kg, 40 mg/kg) could improve depression symptoms. Cytokines interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α in hippocampus presented significant decreases with exposure to betulinic acid. The expressions of RIP140, p-NF-κBp65, p-IκBα, p-IKKα and p-IKKβ were inhibited with BA (20 mg/kg, 40 mg/kg) treatment according to western blot analysis, while immunohistochemical analysis also exhibited certain alterations of RIP140, p-NF-κBp65. The current results suggested the potential antidepressant-like roles of betulinic acid in the UCMS-induced mouse model via inhibiting brain RIP140 activation.

Keywords: Betulinic acid, depression, RIP140

Introduction

As a chronic psychiatric disorder, depression is widely distributed in the general population and is recognized to be one of the most burdensome diseases of society according to World Health Organization [1, 2]. As is well known, depression is associated with inflammatory processes [3] and oxidative stress [4]. UCMS has long been employed in animal model to elicit mimic depression-like disorder and is considered as a reliable duplication of chronic depression in human [5].

Several studies indicate that inflammation may be involved in the development of depression [6, 7]. The serum levels of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, are often elevated in the major depressive disorder (MDD) [8]. Associations between inflammatory markers and individual depressive symptoms have also been described by substantial studies.

It is acknowledged that receptor-interacting protein 140 (RIP140) plays an important role in the mediation of inflammatory cascade [9]. RIP140, activates pro-inflammatory cytokine generation to regulate the inflammatory progression in macrophages, is reported to interact with the essential transcriptional molecule nuclear factor Kappa B (NF-κB) or cAMP response element binding protein (CREB) to modulate the pro-inflammatory cytokines IL-6 and TNF-α expressions [10]. Using an experimental model of endotoxin tolerance, RIP140 was shown to regulate the productions of NF-κB dependent pro-inflammatory cytokines [11]. Several studies indicate RIP140 played an important role in metabolic diseases [13]. But there are few reports about its role in depression.

Betulinic acid (BA, 3β-hydroxyup-20(29)-en-28-oic acid), is a pentacyclic triterpene prepared from betulin obtained from white-barked birch trees [13]. The compound is mainly known for

its anti-tumor and anti-inflammatory activities [14], which indicated that betulinic acid might be implicated in the development of other nervous system diseases, such as depression. The present study was designed to investigate whether BA confers an antidepressant-like effect in mice exposed to unpredictable chronic mild stress (UCMS) and to elucidate its potential mechanism.

Materials and methods

Main reagents and kits

Betulinic acid (BA, purity 98%) was purchased from National Institutes for Food and Drug Control (Beijing, China). Fluoxetine hydrochloride (Flu) was supplied by Changzhou Siyao Pharmaceuticals Co., Ltd. (Changzhou, PR China). Both BA and Flu were dissolved in dimethyl sulfoxide, DMSO. TNF-α, IL-1β and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Nanjing KeyGEN Biotech. CO., Ltd. (Nanjing, China). MDA, SOD, GSH and GPx kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). All primary antibodies were produced by Cell Signaling Technology Inc (Beverly, MA, USA).

Animals

50 male ICR mice (4 weeks, weighing 18-22 g) acquired from Comparative Medicine Centre of Yangzhou University, were housed in an animal facility under standard laboratory condition with a 12 h light/12 h dark cycle circumstance at 22-24°C and humidity of 40-70%. Mice were provided with water and food pellets *ad libitum*. All animal experiments were performed according to protocols approved by China Pharmaceutical University (No. CPU-TCM-2013012) Medicine Animal Care and Use Committee.

Experimental protocol

Mice were randomly assigned to five groups (with 10 in each group) as follows: control group, model group, UCMS + Flu (20 mg/kg) group, UCMS + BA (20 mg/kg) group and UCMS + BA (40 mg/kg) group. Mice were exposed to UCMS for 6 consecutive weeks. The UCMS procedure was performed as previously described [15] with minor modification. After one week adaptation period, mice were subjected to the stressors for six weeks as follows: (1) water deprivation (24 h), (2) food deprivation (24 h), (3) overnight illumination, (4) cage tilting (45°), (5) damp sawdust (200 ml of water in 100 g of sawdust bedding), (6) exposure to a foreign object, (7) inversion of the light/dark cycle, (8) overhang (10 min), (9) exposure to an empty bottle, (10) tail pinch (1 min, 1 cm from the beginning of the tail), (11) oscillation (5 min) and (12) white noise. All the procedures were randomly organized in order to ensure the unpredictable characteristic of the experiment. Control group were undisturbed except for necessary housekeeping procedures. The frequency of stressors was conducted as previously described [16]. Flu and BA were intragastrically administered once a day for 3 weeks from the fourth week. Mice in the control and CUMS model groups received equal volumes of DMSO. Behavior tests were carried out after the last drug administration.

Behavioral evaluation

Sucrose preference test (SPT): Following stimulation were given to mice: (1) water and food deprivation for 24 h, (2) the choice to drink for 12 h from two bottles filling with sucrose solution (1% w/v) and water respectively. To avoid the influence of objective conditions to the experiment, the distance of two bottles to the mice in the cage was the same and the positions of them were switched after 6 h. The final consumption was assessed by weighing the bottles.

$$SPT = (\text{sucrose intake (g)}/(\text{sucrose intake (g)} + \text{water intake (g)})) \times 100$$

Open field test (OFT): The present apparatus and testing procedures were similar to those used previously [17]. The observation cage (40 × 60 × 50 cm) was divided into 12 equal squares and the amount of panes was recorded. The mice were put in the center of the apparatus to acclimatize the environment before the test. The amount of squares crossed, rearing and grooming behaviors were recorded for 4 min.

Forced swimming test (FST): The forced swimming test was conduct according to the conventional method described previously [18] 6 weeks post UCMS challenge with minor modifications. Mice were individually placed in an

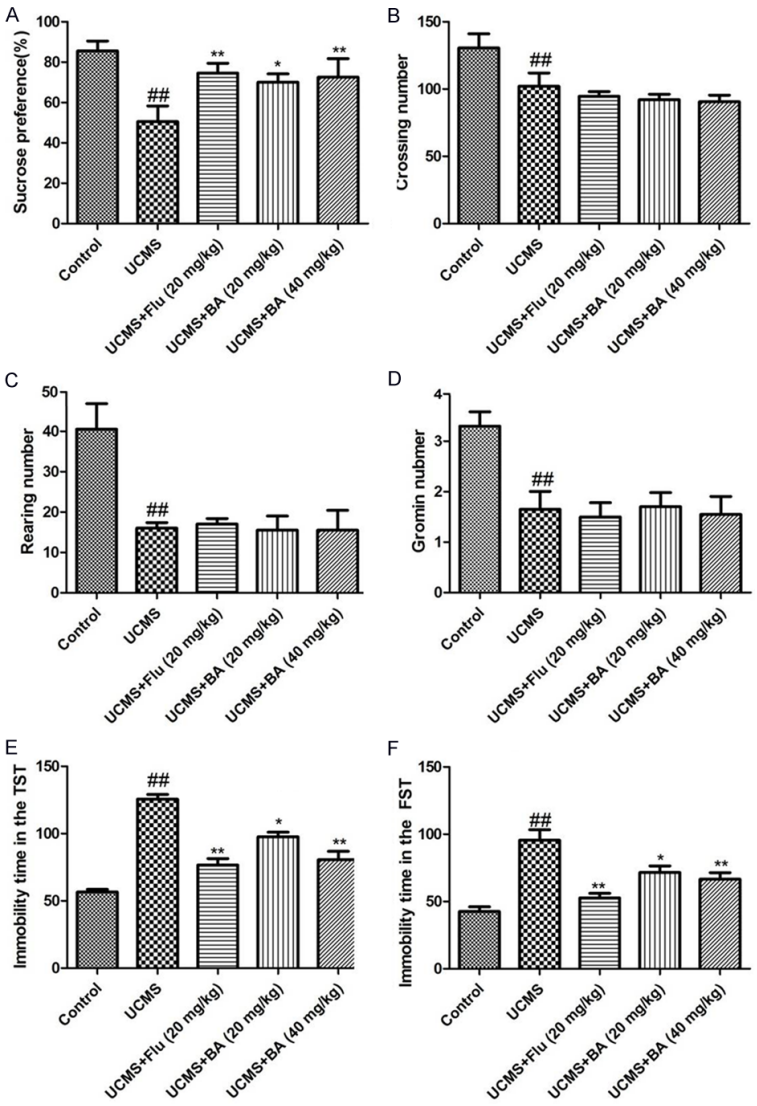

Figure 1. Effects of BA on sucrose consumption (A) in the SPT, the numbers of crossings (B), rearings (C) and grooming (D) in OFT and immobility time in the TST (E) and FST (F). Values are expressed as mean ± SD. Compared with control: #P<0.05, ##P<0.01; Compared with model: *P<0.05, **P<0.01.

open cylindrical container (diameter = 14 cm, height = 20 cm) containing water up to a height of 12 cm at 25 ± 1°C and forced to swim for 6 min. The total duration of immobility time was recorded during the last 4 min period by two independent observers blinded to the experiment. The amount of time spent by the mice floating in the water without struggling and escape-oriented behaviors was defined as immobility time.

Tail suspension test (TST): The tail suspension test was conduct according to the conventional method described previously [19] 6 weeks post UCMS challenge with slight modifications. Every mouse both acoustically and visually separated was individually suspended for 6 min with 50 cm above the floor by adhesive tape (approximately 2 cm from the end). The immobility period was measured for the last 4 min by two independent observers blinded to the experiment. The amount of time spent by the mice remaining completely motionless was defined as immobility time.

Cytokine measurement

Blood samples were harvested from orbit and were centrifuged at 3500 rpm for 10 min to collect the serum. The concentrations of IL-6, IL-1β and TNF-α in serum were detected by ELISA kit according to the manufacturer's instructions. The absorbance of each well was read at 450 nm with a microplate spectrophotometer.

Western blot analysis

Proteins of hippocampus tissues (100 mg) were extracted with lysis buffer (RIPA with protease and phosphatase inhibitor) for 30 min on ice respectively and then centrifuged at 12000 rpm for 5 min at 4°C to remove the debris. Total protein concentration was detected using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Nanjing, China). The samples were loaded on SDS-polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk in Trisbuffer saline and incubated at 4°C overnight with separate primary antibodies, anti-RIP140 (1:1000), anti-NF-κBp65 (1:500), anti-p-NF-κBp65 (1:500), anti-IκBα (1:1000), anti-p-IκBα (1:1000) and anti-IKKα (1:500), anti-p-IKKα (1:500), anti-IKKβ (1:1000), anti-p-IKKβ (1:1000) anti-GAPDH (1:1000). After washing three times with

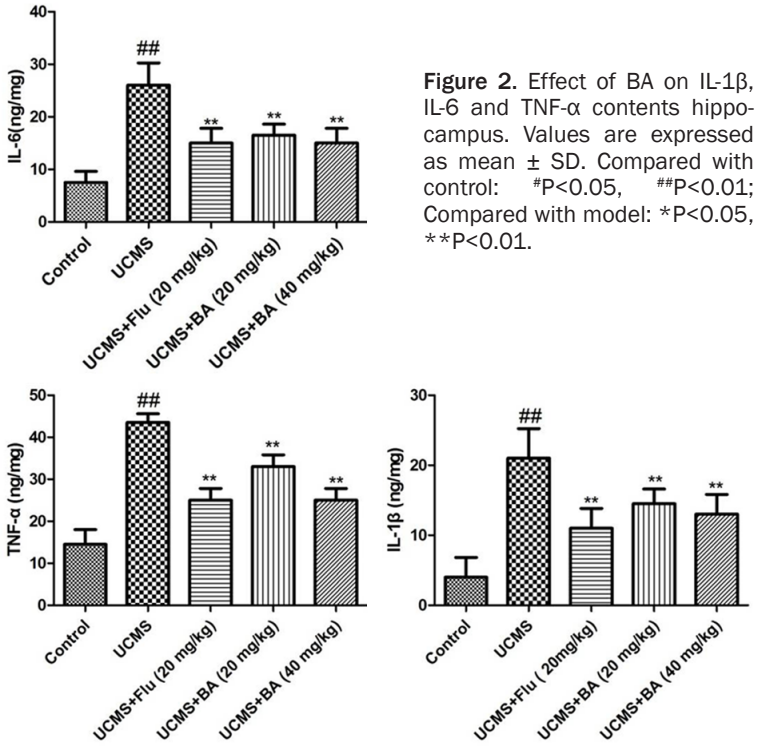

Figure 2. Effect of BA on IL-1β, IL-6 and TNF-α contents hippocampus. Values are expressed as mean ± SD. Compared with control: #P<0.05, ##P<0.01; Compared with model: *P<0.05, **P<0.01.

Tris-buffered saline-Tween-20, themembranes were incubated with secondary antibody (1: 12,000) for 1.5 h at room temperature. The bands were visualized by usingenhanced chemiluminescence detection reagents and a gel imaging system.

Statistical analysis

The data in the figures were expressed as means ± SDs. Assessment between groups were analyzed by one-way analysis of variance (ANOVA) with Tukey multiple comparison test. All data were processed with Graphpad, while $p<0.05$ was considered significant difference.

Results

BA ameliorates depression-related behaviors in the UCMS mouse model Effects of BA on sucrose consumption in the SPT

As one of the most major characteristics of depression, anhedonia can be effectively reflected by the decreased consumption of sucrose solution. As shown in **Figure 1A**, mice with UCMS stimulation alone showed decreased sucrose preference when it is compared with non-UCMS treated ones. BA (20 mg/kg, 40 mg/kg) and Flu (20 mg/kg) treatments for 3 weeks remarkably alleviated the UCMS-induced sucrose preference reduction at the end of the 6 week UCMS challenge.

Effects of BA on locomotor activity in the OFT

Reduced locomotor activity is another core symptom of depression. As shown in **Figure 1B-D**, mice exposed to UCMS showed obvious decreased amounts of crossing rearing and grooming in contrast with those without exposure to UCMS. Intriguingly, there was no significant difference between the groups of BA (20 mg/kg, 40 mg/kg), Flu (20 mg/kg) and control group, which was exhibited in crossings, rearing and grooming numbers.

Effects of BA on immobility time in the TST and FST

As shown in **Figure 1E**, **1F**, the immobility time in the TST and FST were recorded to measure the depressive-like behavior. Mice in the UCMS group displayed obvious increases in immobility duration during TST and FST versus the control group. As expected, Flu (20 mg/kg) and BA (20 mg/kg, 40 mg/kg) markedly reverse the increase in the helpless behavior compared with the UCMS treatment group, suggesting its effect against depressive-like behavior.

Effects of BA on cytokines production

The anti-inflammatory effect of BA was partially evidenced by the detection of cytokines IL-6, IL-1β and TNF-α. The levels of them were dramatically increased versus those in the non-UCMS, respectively. BA (20 mg/kg, 40 mg/kg) effectively suppressed UCMS-induced increases in the levels of IL-1β, IL-6 and TNF-α in hippocampus (**Figure 2**).

Effects of BA on the RIP140/NF-κB-related proteins

Western blot analysis showed the up-regulation of RIP140, p-NF-κBp65, p-IκBα, p-IKKα and p-IKKβ in mice exposed to UCMS with GAPDH, NF-κBp65, IκBα, IKKα and IKKβ expressions as

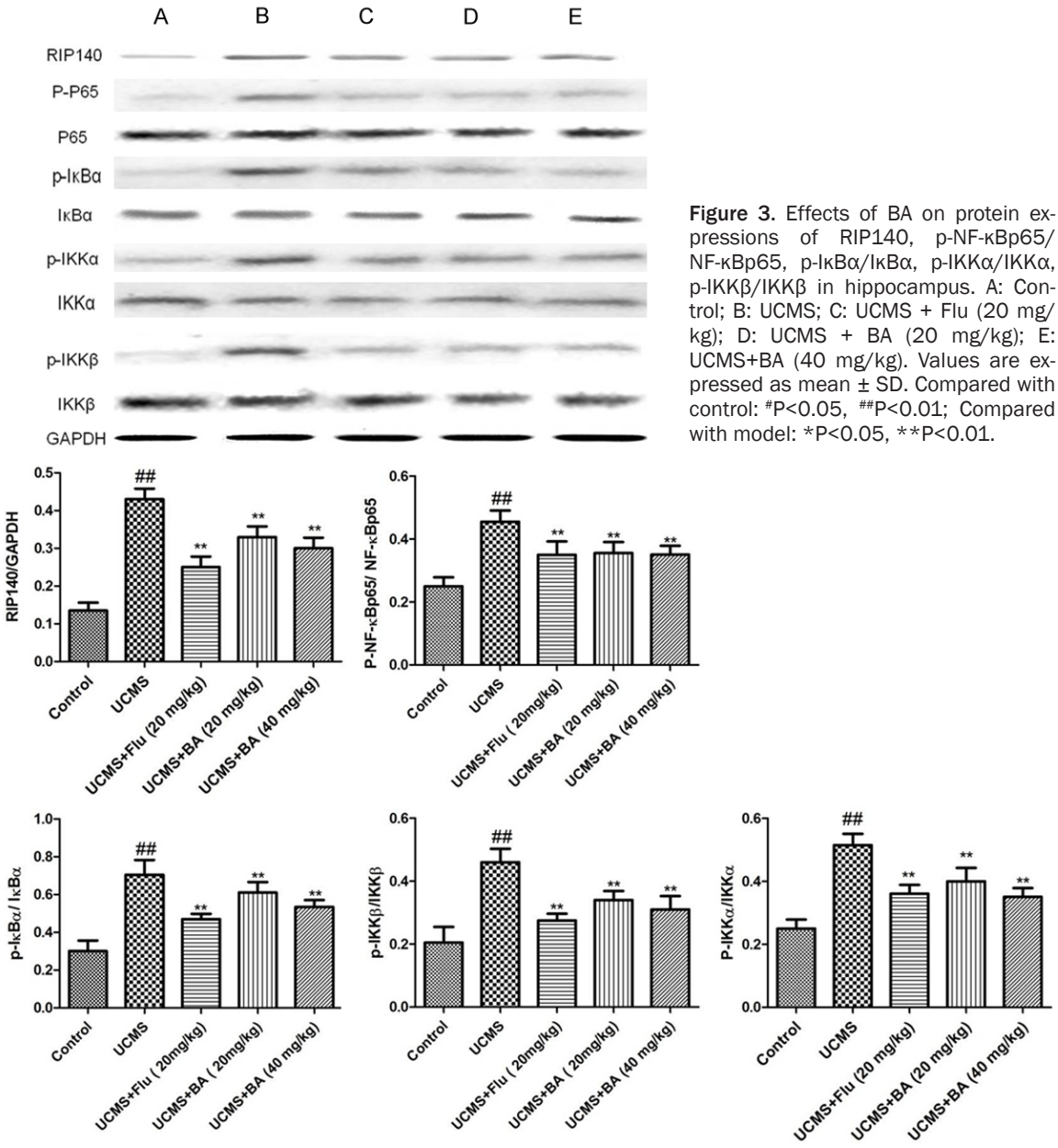

Figure 3. Effects of BA on protein expressions of RIP140, p-NF-κBp65/NF-κBp65, p-IκBα/IκBα, p-IKKα/IKKα, p-IKKβ/IKKβ in hippocampus. A: Control; B: UCMS; C: UCMS + Flu (20 mg/kg); D: UCMS + BA (20 mg/kg); E: UCMS+BA (40 mg/kg). Values are expressed as mean ± SD. Compared with control: #P<0.05, ##P<0.01; Compared with model: *P<0.05, **P<0.01.

internal controls respectively, while different degrees of down-regulation of them were observed in BA (20 mg/kg, 40 mg/kg) group, respectively (**Figure 3**).

Discussion

The rodent preference for sweet solutions is a core symptom of major depression [20-22], which is hypothesized to represent anhedonia. This anhedonic-like-behavior is commonly investigated in mice via the SPT experiment. Additionally, activity state change of the patients is often accompanied with depression, which can be regarded as the index to observe depression. Open field test is also widely used to evaluate locomotor and exploratory behaviors in experimental animals. Meanwhile, TST and FST are the most commonly usual methods for assessing depression and anxiety, which is partially attributed to their high predictive validity [23]. Wherein, TST is also extensively used to screen the antidepressant-like property of novel drugs and on behalf of a failure of persistence in the escape-directed behavior [24]. In line with previous literatures, our findings showed UCMS increased the immobility time in the FST and TST, reduced

sucrose intake volume and the number of squares crossed rearing and grooming. BA treatment recovered the sucrose preference and reduced the immobility time in the FST and TST, but the number of squares crossed, rearing and grooming showed no changes, which revealed that BA might function as an antidepressant drug without central nervous system (CNS) excitability.

Emerging evidences from clinical or preclinical stages are suggesting that the inhibition of cytokine productions can lead to a reduction of inflammation-induced depressive-like behavior. The concentrations of pro-inflammatory cytokines, including IL-6, IL-1β and TNF-α, were measured in hippocampus since it play a major role in the modulation of emotional behavior and neuroendocrine. In turn, cytokines, especially IL-1, are proved to be involved in the regulation of neuroendocrine systems [25]. The obtained data exhibited that the levels of IL-6, IL-1β and TNF-α were elevated in different degrees in UCMS-induced mice. ELISA assay declared the inhibitory effect of BA on the production of cytokines IL-6, IL-1β and TNF-α in hippocampus, indicating that the antidepressant-like property of BA might be partially attributed to its anti-inflammatory effect.

Neuroinflammation has been reported to play an essential role in depression. As previous studies suggested, related signaling path of inflammation has been highly associated with various diseases [26, 27]. Thus, we next explored the signal transduction pathway illustrating the molecular link between inflammation and depression. It is acknowledged that receptor-interacting protein 140 (RIP140) is a major factor accounting for the mediation of inflammatory cascade. RIP140, activating pro-inflammatory cytokine generation to regulate the inflammatory progression in macrophages, is reported to further interact with the essential transcriptional molecule nuclear factor Kappa B (NF-κB) or cAMP response element binding protein (CREB). Moreover, the phosphorylation of IκB triggers the activation of NF-κB that would contribute to the transcriptions of many pro-inflammatory genes and the expressions of inflammatory cytokines including TNF-α and IL-6 [28-30]. In the present study, UCMS-induced mice which were given betulinic acid treatment displayed the decrease of RIP140 and the decrease of p-NF-κBp65, p-IκBα, p-IKKα and p-IKKβ levels in hippocampus, confirming our hypothesis that betulinic acid may exert antidepressant effects via regulating RIP140/NF-κB signaling.

In conclusion, the present work provides a novel mechanism for BA to treat depression. This mechanism was related to inhibit RIP-140/NF-κB pathway.

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Disclosure of conflict of interest

None.

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References

[1] Patten SB. Major depression prevalence is very high, but the syndrome is a poor proxy for community populations' clinical treatment needs. Can J Psychiatry 2008; 53: 411-419.

[2] Deng XY, Li HY, Chen JJ, Li RP, Qu R, Fu Q, Ma SP. Thymol produces an antidepressant-like effect in a chronic unpredictable mild stress model of depression in mice. Behav Brain Res 2015; 291: 12-19.

[3] Al-Hakeim HK, Al-Rammahi DA, Al-Dujaili AH. IL-6, IL-18, sIL-2R, and TNFalpha proinflammatory markers in depression and schizophrenia patients who are free of overt inflammation. J Affect Disord 2015; 182: 106-114.

[4] Kumar B, Arora V, Kuhad A, Chopra K. Vaccinium myrtillus ameliorates unpredictable chronic mild stress induced depression: possible involvement of nitric oxide pathway. Phytother Res 2012; 26: 488-497.

[5] Ma Z, Ji W, Qu R, Wang M, Yang W, Zhan Z, Fu Q, Ma S. Metabonomic study on the antidepressant-like effects of banxia houpu decoction and its action mechanism. Evid Based Complement Alternat Med 2013; 2013: 213739.

[6] Tianzhu Z, Shihai Y, Juan D. Antidepressant-like effects of cordycepin in a mice model of chronic unpredictable mild stress. Evid Based Complement Alternat Med 2014; 2014: 438506.

[7] Zhu L, Wei T, Gao J, Chang X, He H, Miao M, Yan T. Salidroside attenuates lipopolysaccha-

ride (LPS) induced serum cytokines and depressive-like behavior in mice. Neurosci Lett 2015; 606: 1-6.
[8] Hannestad J, DellaGioia N, Bloch M. The effect of antidepressant medication treatment on serum levels of inflammatory cytokines: a meta-analysis. Neuropsychopharmacology 2011; 36: 2452-2459.
[9] Wei LN, Farooqui M, Hu X. Ligand-dependent formation of retinoid receptors, receptor-interacting protein 140 (RIP140), and histone deacetylase complex is mediated by a novel receptor-interacting motif of RIP140. J Biol Chem 2001; 276: 16107-16112.
[10] Zschiedrich I, Hardeland U, Krones-Herzig A, Berriel Diaz M, Vegiopoulos A, Muggenburg J, Sombroek D, Hofmann TG, Zawatzky R, Yu X, Gretz N, Christian M, White R, Parker MG, Herzig S. Coactivator function of RIP140 for NFkappaB/RelA-dependent cytokine gene expression. Blood 2008; 112: 264-276.
[11] Ho PC, Tsui YC, Feng X, Greaves DR, Wei LN. NF-kappaB-mediated degradation of the coactivator RIP140 regulates inflammatory responses and contributes to endotoxin tolerance. Nat Immunol 2012; 13: 379-386.
[12] Seth A, Steel JH, Nichol D, Pocock V, Kumaran MK, Fritah A, Mobberley M, Ryder TA, Rowlerson A, Scott J, Poutanen M, White R, Parker M. The transcriptional corepressor RIP140 regulates oxidative metabolism in skeletal muscle. Cell Metab 2007; 6: 236-245.
[13] Soica C, Danciu C, Savoiu-Balint G, Borcan F, Ambrus R, Zupko I, Bojin F, Coricovac D, Ciurlea S, Avram S, Dehelean CA, Olariu T, Matusz P. Betulinic acid in complex with a gamma-cyclodextrin derivative decreases proliferation and in vivo tumor development of non-metastatic and metastatic B164A5 cells. Int J Mol Sci 2014; 15: 8235-8255.
[14] Nick A, Wright AD, Rali T, Sticher O. Antibacterial triterpenoids from dillenia papuana and their structure-activity relationships. Phytochemistry 1995; 40: 1691-1695.
[15] Willner P, Towell A, Sampson D, Sophokleous S, Muscat R. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology 1987; 93: 358-364.
[16] Li M, Fu Q, Li Y, Li S, Xue J, Ma S. Emodin opposes chronic unpredictable mild stress induced depressive-like behavior in mice by upregulating the levels of hippocampal glucocorticoid receptor and brain-derived neurotrophic factor. Fitoterapia 2014; 98: 1-10.
[17] Xue J, Li H, Deng X, Ma Z, Fu Q, Ma S. L-Menthone confers antidepressant-like effects in an unpredictable chronic mild stress mouse model via NLRP3 inflammasome-mediated inflammatory cytokines and central neurotransmitters. Pharmacol Biochem Behav 2015; 134: 42-48.
[18] Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 1977; 229: 327-336.
[19] Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology (Berl) 1985; 85: 367-370.
[20] Li J, Han B, Ma X, Qi S. The effects of propofol on hippocampal caspase-3 and Bcl-2 expression following forebrain ischemia-reperfusion in rats. Brain Res 2010; 1356: 11-23.
[21] Sibille E, Wang Y, Joeyen-Waldorf J, Gaiteri C, Surget A, Oh S, Belzung C, Tseng GC, Lewis DA. A molecular signature of depression in the amygdala. Am J Psychiatry 2009; 166: 1011-1024.
[22] Willner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology 1997; 134: 319-329.
[23] Peng WH, Lo KL, Lee YH, Hung TH, Lin YC. Berberine produces antidepressant-like effects in the forced swim test and in the tail suspension test in mice. Life Sci 2007; 81: 933-938.
[24] Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 2005; 29: 571-625.
[25] Liu B, Xu C, Wu X, Liu F, Du Y, Sun J, Tao J, Dong J. Icariin exerts an antidepressant effect in an unpredictable chronic mild stress model of depression in rats and is associated with the regulation of hippocampal neuroinflammation. Neuroscience 2015; 294: 193-205.
[26] Wang Q, Wen R, Lin Q, Wang N, Lu P, Zhu X. Wogonoside shows Antifibrotic effects in an experimental regression model of hepatic fibrosis. Dig Dis Sci 2015; 60: 3329-3339.
[27] Jiang W, Luo F, Lu Q, Liu J, Li P, Wang X, Fu Y, Hao K, Yan T, Ding X. The protective effect of Trillin LPS-induced acute lung injury by the regulations of inflammation and oxidative state. Chem Biol Interact 2016; 243: 127-134.
[28] Chen T, Mou Y, Tan J, Wei L, Qiao Y, Wei T, Xiang P, Peng S, Zhang Y, Huang Z, Ji H. The protective effect of CDDO-Me on lipopolysaccharide-induced acute lung injury in mice. Int Immunopharmacol 2015; 25: 55-64.
[29] Chang X, Luo F, Jiang W, Zhu L, Gao J, He H, Wei T, Gong S, Yan T. Protective activity of salidroside against ethanol-induced gastric ulcer via the MAPK/NF-kappaB pathway in vivo and in vitro. Int Immunopharmacol 2015; 28: 604-615.
[30] Jing W, Chunhua M, Shumin W. Effects of acteoside on lipopolysaccharide-induced inflammation in acute lung injury via regulation of NF-kappaB pathway in vivo and in vitro. Toxicol Appl Pharmacol 2015; 285: 128-135.