Brazilin Treatment Produces Antidepressant- and Anxiolytic-Like Effects in Mice

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Increasing evidence shows depression relevant to oxidative stress and inflammation. Anti-inflammatory strategies or antioxidants have led to the development of new antidepressants. Brazilin is a natural product from the Chinese traditional medicine Caesalpinia sappan L., exerting anti-inflammatory, antioxidant, anti-platelet concentration, and anti-cancer effects. While the antidepressant effect of brazilin is largely unknown. In present study, we investigated the effects of brazilin on H2O2-induced oxidative injury in PC12 cells and on depression- and anxiety-like behaviors of chronically mild stressed (CMS)-induced depression mice. It was found that brazilin pre-treatment (both 10 and 20µM) significantly increased cell viability and decreased cell apoptosis in H2O2-treated PC12 cells. Furthermore, repetitive administration of brazilin to CMS-induced depression mice by intraperitoneal injection (10mg/kg) made the mice significantly lose their latency of feeding in novelty-suppressed feeding test (NSF), have more the sucrose preference in sucrose preference test (SPT), and more time spent in the central zone without affecting their crossing activity in open field test (OFT). These results suggested that brazilin can play a role in antidepressant and anxiolytic-like behaviors for CMS-induced depression mice probably through inhibiting the oxidative stress. Therefore, brazilin is worth to be further explored for treating depressive and anxiety disorders.

Key words brazilin; depression; anxiety; chronic mild stress; oxidative stress

INTRODUCTION

Alarming number of individuals suffering depression and anxiety due to stresses of modern life. Stress is a threat to the homeostasis of the organism and may cause physiological and behavioral changes to maintain equilibrium. Studies have indicated that chronic stress is a critical risk factor for psychiatric disorders like depression and post-traumatic stress disorder (PTSD). Both prefrontal cortex and hippocampus are brain regions controlling emotion, mood, and cognition for response to chronic stress. The synaptic loss in these two regions is associated with depression. Depression is a common psychiatric disease with a lifetime prevalence and causes major financial burden. It could make the susceptible person suffer great mental anguish and function poorly in daily life, even leading to suicide. Reports from WHO indicated that close to eight-hundred thousand people commit suicide every year. To elucidate the underlying mechanism of depression and to develop antidepressants are the main goals of neuroscience worldwide.

The present antidepressants, such as selective serotonin reuptake inhibitors and tricyclic antidepressants, are with low efficiency and series of adverse effects. It is urgent to find novel, relatively safer and more effective antidepressants for depression treatment in the clinic. The molecular mechanisms underlying depression are still poorly understood. Recently, several novel theories about pathology of depression, including increased excessive inflammation and oxidative stress injuries, have been provided and have attracted the attention for the development of antidepressants. Overproduction of reactive oxygen species (ROS) induced by stress causes brain oxidative damage, which would be a potential treatment target for depression. Some natural compound extracted from herbs that used in traditional Chinese medicine was reported to have significant antidepressant activities, comparable efficiency to existing antidepressants with less side effects. Our previous studies reported that several extracts from natural herb, such as sulforaphane, cupadens A and Suhexiang have antidepressant- and anxiolytic-like effects with no significant side effects in mice.

Sumu, part of the plant Caesalpinia sappan L. and a classic medicine used in traditional Chinese medicine, has been widely used in China for its circulatory promoting and anti-inflammation effects. Brazilin, the main homoisoflavonoid constituent from Sumu heartwood (molecular structure in Fig. 1A), is well known for the natural red color dye for staining and has anti-oxidative stress, anti-inflammatory, anti-platelet concentration, and anti-cancer effects.

Considering the relationship of inflammation, oxidative stress and depression, in the present study, we evaluated the
potential neuroprotective effects of brazillin on H2O2-induced rat pheochromocytoma (PC12) cell injury and chronic mild stress (CMS) mice model.

MATERIALS AND METHODS

**Animals** Seven-week old male ICR mice was purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China) and were maintained at 23–25°C under 50 ± 5% humidity on artificial 12 h/12 h light and dark cycle (light on at 8:00 p.m., light off at 8:00 a.m.) with ad libitum access to water and food. After acclimating for 5 d the mice were divided into 5 groups: Naïve, CMS+Vehicle, CMS+Brazillin (3 mg/kg), CMS+Brazilin (10 mg/kg) and CMS+Fluoxetine (10 mg/kg). All animal experiments were conducted approved by Animal Care and Use Committee of Hebei Medical University.

**Drugs and Reagents** Brazillin (CAS No. 474-07-7, Fig. 1) extracted and purified from dried *Caesalpinia sappan* L. as reported was purchased from Yuanye Bio-Technology (Shanghai, China). Fetal bovine serum (FBS) was purchased from ScienCell (U.S.A.). High glucose Dulbecco’s modified Eagle’s medium (DMEM) medium was purchased from GEL life science (Logan, UT, U.S.A.). All chemicals and reagents were of analytical grade.

**Cell Culture and Treatment** PC12 cells were cultured in high glucose DMEM with 10% FBS at 37°C in a humidified incubator containing 5% CO2. For treatment, the cells were seeded onto 96-well culture plate at 5 × 10^4 cells/well and cultured until approximately 70–80% confluent. One hour after brazillin treatment, the cells were incubated in culture medium with H2O2 (200 μM) for another 24 h.

**Cell Viability Assay** The cell viability was evaluated by MTS assay using CellTiter 96 O AQueous One Solution cell proliferation kit (Promega, Madison, WI, U.S.A.) according to the manufacturer’s protocol. At the end of treatment, the cells in each well of 96-well plate were washed with phosphate buffered saline (PBS) once and incubated with 100 μL serum-free media and 20 μL CellTiter 96 O AQueous One Solution at 37°C for 3 h in the dark. Then the absorbance at 490 nm (A_490) was measured. The viability was represented as the percentage of treated cell A_490 to control cell A_490.

**Flow Cytometry** Cell apoptosis was determined by flow cytometry assay using Annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI) staining with kit (Wanlei Biotechnology, China). The PC12 cells were planted into 6-well plate and treated with different factors. At the end of treatment, the cells were washed twice with cold PBS and suspended in 1x binding buffer. Then FITC annexin V and PI were used to stain the cells for 15 min at 25°C in the dark. The stained cells were analyzed by flow cytometry (Guava Technology).

**Introduction of CMS in Mice and Treatment** CMS model of depression in mice was conducted based on our previous studies. Mice were treated once a day for 28 d with two randomly chosen stressors from following: 2 h-restraint, 5 min-forced cold swimming, 24 h-water deprivation, 12 h-food deprivation, 24 h-tilted cages, 24 h-soiled bedding, 24 h-light/dark cycle reversal, 1 min-tail clamping, 24 h-crowded space and 24 h-bedding deprivation. And from the 21st day of their 28-d treatment, the CMS mice were randomly divided into 4 sub-groups (CMS + Vehicle, CMS + Brazilin 3 mg/kg, CMS + Brazilin 10 mg/kg and CMS + Fluoxetine) and intraperitoneally injected with saline (10 mL/kg), brazilin (3 mg/kg), brazilin (10 mg/kg) or fluoxetine (10 mg/kg) daily for continuous 7 d. Meanwhile, mice in Naïve group were raised in their home cage for 28 d without any stressor treatment. At 24 h after the last administration, behavioral tests were performed.

**Open Field Test (OFT)** In the OFT, mice were placed in the center of an open field box (40 × 40 × 35 cm), lighted 20 lx intensity and observed for their locomotion according to the literature procedures. The time spent (second) in the central zone and total distance traveled (cm) by mice were recorded by video tracking system (SMART 3.0, Panlab, Spain) for 5-min.

**Novelty Suppressed Feeding Test (NSF)** NSF was performed as described by our previous report. Mice were deprived of food for 24 h and then individually placed in the corner of a square arena (40 × 40 × 35 cm) that contained a small pellet of food in the arena center. After finishing the food, mice were transferred back to their home cages. The latency to approach the food (maximum for 300 s) and food consumption in home cage were recorded to evaluate extent of anxiety and appetite of the mice.

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**Fig. 1.** Chemical Structure of Brazilin and Purity Analysis by HPLC (A) Chemical structure of brazilin. (B) The purity of brazilin (95%, t_R = 20.3 min) is detected by HPLC with methanol–water (30:70) as a mobile phase with DAD detector (1.0 mL/min).
Sucrose Preference Test (SPT) SPT was performed according to the previous description. Mice were given 24 h of acclimation to the two bottles of 1% sucrose solution and followed by water deprivation for 24 h prior to SPT. Then, mice were placed in individual cages and given free access to two bottles, one containing of water and another containing 1% sucrose solution for 24 h. At the end of SPT, the amount of water and sucrose consumed for 24 h were measured. The sucrose preference was expressed as the percentage of volume of sucrose solution consumed over the total liquid taken.

Statistical Analysis Results were expressed as means ± standard deviation (S.D.). Data were analyzed using SPSS13.0 software. One-way ANOVA was used to test intergroup differences for multiple group comparison. p < 0.05 was considered statistically significant.

RESULTS

Brazilin Reduced H₂O₂-Induced Cytotoxicity in PC12 Cells Treatment with over 200 μM H₂O₂ for 24 h caused the PC12 cell viability reduced from 100% (control) to 37% of the control value (Fig. 2A). While treatment with brazilin at the concentration below 40 μM for 24 h did not show significant cytotoxic effect on PC12 cells (Fig. 2B). Pre-treatment with brazilin (both 10 and 20 μM) 1 h significantly attenuated 200 μM H₂O₂-induced decrease in cell viability (47.83 and 91.51% of the control value, respectively) (Fig. 2C) and in cell proliferation (Fig. 2D).

Brazilin Antagonized H₂O₂-Induced Apoptosis in PC12 Cells As shown in Fig. 3, treatment with 200 μM H₂O₂ for 24 h caused considerable apoptosis (85.02 ± 2.71%) and necrosis (11.04 ± 1.16%) in PC12 cells compared with control group (apoptosis: 27.07 ± 5.76%, necrosis: 4.61 ± 0.16%). And the H₂O₂-induced apoptosis was blocked by pre-incubation for 1 h with brazilin of 10 μM (apoptosis: 62.20 ± 8.30%, necrosis: 6.38 ± 0.81%) and 20 μM (apoptosis: 55.77 ± 10.94%, necrosis: 6.01 ± 0.98%). These data suggested that brazilin showed protective effect against H₂O₂-induced apoptosis and necrosis (Fig. 3).

Brazilin Alleviated the CMS Induced Depressive- and Anxiety-Like Behaviors in Adult Mice To further evaluate the effects of brazilin on mice suffering chronic stress, chronic mild stress (CMS) mice model were used in this experiment as shown in Fig. 4A. The OFT and NSF were performed to evaluate the anxiety state of mice. SPT was used to reveal the adhedonia behavior of mice.

In OFT, CMS mice spent less time in the central zone (F<sub>1,19</sub> = 13.296, p < 0.005, Fig. 4B) compared with naïve mice. The brazilin (3 mg/kg) treatment increased the time spent in the central zone in the OFT (F<sub>3,35</sub> = 2.052, p < 0.05, Fig. 4B) compared to the mice in CMS + VEH group. While the treatment with 10 mg/kg of fluoxetine and brazilin also showed the time spent in the central zone on the rise though no statistical significance. Neither fluoxetine nor brazilin treatment showed significant effect on the crossing activities (Fig. 4C).

In NSF, CMS mice exerted a prolonged latency to feeding (F<sub>1,19</sub> = 18.839, p < 0.001, Fig. 4D) compared with mice in naïve group. Treatment with 10 mg/kg of brazilin and fluoxetine decreased the prolonged latency to feeding significantly (F<sub>3,35</sub> = 3.065, p < 0.05, respectively, Fig. 4D) without influencing the total feeding in home cage (Fig. 4E).

In SPT, CMS mice showed a decrease in sucrose preference (F<sub>1,19</sub> = 4.094, p < 0.01, Fig. 4F), which exhibited depressive-like phenotype. And the treatment with both fluoxetine and brazilin (10 mg/kg) significantly increased sucrose preference (F<sub>3,35</sub> = 9.776, p < 0.01 respectively, Fig. 4F) of CMS mice, without effects on the total water intake (Fig. 4G). No significant difference in sucrose preference occurred between mice.
in braziliin and fluoxetine treatment groups, showing that braziliin could exert the same anti-depressant effect as fluoxetine. Taken together, these results show that braziliin treatment could attenuate CMS-induced depressive- and anxiety-like behaviors.

DISCUSSION

Experiments both \textit{in vivo} and \textit{in vitro} were designed in the present study. In order to imitate the oxidative-stress condition in brain of patients with depression, H\textsubscript{2}O\textsubscript{2}-treated PC12 cells firstly were used to test the effect of braziliin. It was found that braziliin pre-treatment (both 10 and 20 \(\mu\)M) significantly attenuated H\textsubscript{2}O\textsubscript{2}-induced decrease in cell viability and H\textsubscript{2}O\textsubscript{2}-induced increase in apoptosis and necrosis in PC12 cells. And the protective effect of braziliin is dose-dependent. Secondly, CMS mice were used to mimic the chronically stressed people for braziliin study. It was found that repeated administration of braziliin (10 mg/kg) significantly decreased the latency to feeding in NSF and increased sucrose preference in SPT of chronic mild stress-induced depression mice. And in OFT of CMS mice, braziliin (3 mg/kg) treatment also significantly increased the time spent in the central zone but not the crossing activity. Interestingly, braziliin showed its antidepressant effect at a higher dose (10 mg/kg) and anxiolytic-like effect at a lower dose (3 mg/kg). It may be a shortcoming of the braziliin as an antidepressant or anxiolytic medicine that exerts different effects at different doses. In conclusion, braziliin has antidepressant- and anxiolytic-like effects in CMS mice, which may be involved in inhibiting the oxidative stress. These data indicate that braziliin is worth to be further explored for treating depressive and anxiety disorders.

A lot of data suggest that oxidative stress is involved in the pathophysiology of depression,\textsuperscript{25–27} anxiety,\textsuperscript{26,28} bipolar disorder,\textsuperscript{28–32} panic disorder\textsuperscript{33,34} and obsessive-compulsive disorder.\textsuperscript{35} Postmortem study showed that the oxidative stress was increased and the anti-oxidant ability was decreased in major depressive disorder (MDD) patients.\textsuperscript{25} Oxidative stress is the condition of imbalance between the production of cytotoxic reactive oxygen species (ROS) and the capacity of antioxidant systems to withstand them.\textsuperscript{36} ROS includes hydrogen peroxide, superoxide anion, hydroxyl radical (HO\textsuperscript{-}), nitric oxide, peroxyl, reactive aldehyde and so on. As signaling molecules, moderate level of ROS plays an important role to maintain normal cell function, while ROS overproduction can cause oxidative damage to cells,\textsuperscript{37} which can further contribute to endothelium dysfunction and increase permeability of blood brain barrier.\textsuperscript{38} Also, ROS can alter blood flow and conse-
quently regulate blood pressure in brain, thus induce changes in microcirculation of brain. In our study, we use moderate concentration of \( \text{H}_2\text{O}_2 \) as an oxidative stressor to incubate PC12 cells for 24 h to mimic chronic mild oxidative stress occurred in brain. \( \text{H}_2\text{O}_2 \) treatment caused the decrease in cell viability and the increase in cell apoptosis and necrosis, which is consistent with previous reports.40,41) It is widely accepted that the present antidepressants have many limitations, low-efficiency, and variety of side effects. A large international cohort study showed that more than half of the adult antidepressant users experienced emotional blunting, suicidality, and withdrawal effects.42) There are increased risks for suicide in younger antidepressant users.43) Depression usually does not come alone. It is reported that about 45–87% of patients with depressive disorder are accompanied with anxiety symptoms.44,45) It is needful to investigate a more effective and safer antidepressant to treat depression as well as anxiety.

Brazilin is the main ingredient of *Caesalpinia sappan* L., distributed in South-east Asia. In traditional Chinese medicine, the central part of *Caesalpinia sappan* L. trunk was used to promote blood circulation and treat wounds without obvious side effects. Evidence has shown that brazilin exhibits multiple functions such as anti-inflammation activity, anti-platelet activity, anti-oxidative activities, preventing hepatotoxicity, and inducing immunological tolerance.20,46–49) It was reported that brazilin can be distributed in brain after intravenous administration of 50 mg/kg brazilin to rats,50 which indicates that brazilin may act directly in brain by passing through the blood brain barrier. Since oxidative stress plays a crucial part in MDD, so we assumed that brazilin may have an anti-depression effect by acting as an anti-oxidative factor. In cell experiment, we found that 24 h brazilin treatment did not cause significant cytotoxicity to PC12 cells with the concentration below 40 \( \mu \text{M} \). Brazilin (both 10 and 20 \( \mu \text{M} \)) pre-treatment significantly attenuated \( \text{H}_2\text{O}_2 \)-induced decrease in cell viability in and antagonized \( \text{H}_2\text{O}_2 \)-induced apoptosis and necrosis in PC12 cells. And the protective effect of brazilin is dose-dependent. These results provide evidence that brazilin could exert anti-oxidative effect on cells, indicating that brazilin may ameliorate the oxidative stress and relieve the depressive and anxiety symptoms.

Extensive data has shown that stress, especially chronic stress, plays a vital role in the development of depression.51) Various depressive- and anxiety-like behaviors in mice model, like the decreased sucrose preference in SPT and the increased latency to feeding in NSF, could be induced by the chronic stress in mice model and can be alleviated by plant extracted compounds (*i.e.*, sulforaphane, tea polyphenol).15,23) To investigate whether brazilin also has the antidepressant-like and anxiolytic effects, CMS-induced depression mice model was carried out and behavioral tests (SPT, OFT and NSF) were conducted. The results showed that continuous 7-d administration of brazilin significantly increased sucrose preference of CMS-treated mice as well as fluoxetine treatment,

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Fig. 4. Brazilin Alleviated the Depressive- and Anxiety-Like Behaviours in Chronically Stressed Mice

(A) Experimental procedure. Brazilin significantly increased the time spent in the central zone (B) without affecting the crossing activity (C) of chronically stressed mice during the NSF test. Brazilin significantly reversed the chronic stress-induced decrease in the sucrose preference (F) without affecting the total intake (G) *\( p < 0.05 \) and **\( p < 0.01 \) versus the Naive group; **\( p < 0.01 \) versus the CMS-treated group. Brazilin alleviated the depressive- and anxiety-like behaviours in chronically stressed mice.
showing that brazilin could exert comparable anti-depressant effect to fluoxetine. Simultaneously, data of OFT and NSF were also revealed that CMS induced anxiety-like behaviors in mice, which could be blocked by repeated brazilin treatment. The present behavioral results indicate that brazilin may prevent depressant- and anxiolytic-like behaviors.

Previous studies on animal models and clinical data have revealed that chronic stress could induce depression and company with excessive oxidation stress and inflammation. Anti-oxidative and anti-inflammatory therapies have attracted more and more attention for development of antidepressants. It was reported that several molecules, such as inducible nitric oxide (iNOS), cyclooxygenases (COX1/COX2), nuclear factor kappa B (NF-kB), and NO, were related to the development of depression and antidepressant treatment. The anti-oxidant and anti-inflammation and other pharmacological activities of Sumu and/or its extracts have been well investigated in vivo and in vitro. The antioxidant activity of brazilin has been explained by different mechanisms, such as prevention of chain initiation, peroxide decomposition, free radicals scavenging and prevention of hydrogen abstraction. Brazilin was reported to exert anti-inflammatory activity by inhibiting NO production, suppressing iNOS activity and inducing of heme oxygenase 1 (HO-1) expression. Combined with these functional molecules mentioned above, the precise mechanism underlying the antidepressant effects of brazilin should be further investigated.

CONCLUSION

In summary, brazilin can significantly attenuate H$_2$O$_2$-induced decrease in cell viability and H$_2$O$_2$-induced increase in apoptosis in PC12 cells. And also brazilin can relieve CMS-induced depressant- and anxiolytic-like behaviors in mice, which is likely by inhibiting the oxidative stress. Our present study offers brazilin, a promising medicine for depression treatment. Further research is needed to see whether brazilin has such effects on human and the underlying molecular mechanism.

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Conflict of Interest The authors declare no conflict of interest.

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REFERENCES


