Journal of Exploratory Research in Pharmacology **2021** vol. 6(4) | 148–157
DOI: 10.14218/JERP.2021.00016

Original Article

Treatment with Gaoziban Tablet Ameliorates Depression by Promoting GSK-3β Phosphorylation to Enhance the Wnt/β-catenin Activation in the Hippocampus of Rats

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Received: June 02, 2021 | **Revised:** August 06, 2021 | **Accepted:** August 16, 2021 | **Published:** September 9, 2021

Abstract

Background and objectives: Depression is a severe and recurrent mental disease and contributes to the global disease burden. However, there are limited effective treatments for depression. This study evaluated the effect of a compound Gaoziban tablet (CGZBT) on depression and explored its potential mechanisms that underlie its action in rats.

Methods: CGZBT was analyzed by high-performance liquid chromatography. Sprague-Dawley rats were randomized into the control, chronic unpredictable mild stress (CUMS), CUMS + 0.4 g/kg CGZBT, CUMS + 0.8 g/kg CGZBT, CUMS + 1.6 g/kg CGZBT, and CUMS + 10 mg/kg fluoxetine (Flu) groups. CGZBT was administered once a day for 14 days, which started on day 28 after the induction of CUMS. Animal behaviors were assessed using the sucrose preference test, forced swimming test, and open field test weekly. The levels of neurotransmitters were identified by liquid chromatography-mass spectrometry, cytokines were quantified by enzyme-linked immunosorbent assay, and CA1 cells were counted after hematoxylin-eosin staining. The expression levels of the proteins of interest were assessed using immunohistochemistry and western blotting.

Results: Compared with the controls, the administration of CGZBT significantly increased the levels of norepinephrine, 5-hydroxytryptamine, and 5-hydroxyindoleacetic acid and serum interleukin (IL) 4 and IL10, but decreased tumor necrosis factor-alpha (TNF-α), IL1β, and IL6 in rats. The number of cells in the hippocampal CA1 area increased. In addition, CGZBT reduced the levels of Axin and adenomatous polyposis coli expression in the hippocampus and significantly upregulated the levels of Wnt1, β-catenin expression, and glycogen synthase kinase-3β (GSK-3β) phosphorylation in the brains of rats.

Conclusions: Our results demonstrated that CGZBT significantly ameliorated depression by promoting GSK-3β phosphorylation to enhance Wnt/β-catenin activation. Our findings might provide a basis for the clinical application of CGZBT.

Keywords: Medicine; Chinese traditional; Depression; Proteins; Behavior and behavior mechanisms.

Abbreviations: 5-HIAA, 5-Hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; APC, adenomatous polyposis coli; CGZBT, compound gaoziban tablet; CUMS, chronic unpredictable mild stress; ELISA, enzyme-linked immunosorbent assay; Flu, Fluoxetine; FST, forced swimming test; GSK-3β, glycogen synthase kinase-3β; H&E, hematoxylin-eosin.; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; NE, norepinephrine; OPT, open field test; SPT, sucrose preference test; TNF-α, tumor necrosis factor-alpha..

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How to cite this article: Zou XS, Yin HL, Shi L, Li HP, Wang MH, Song WC, *et al*. Treatment with Gaoziban Tablet Ameliorates Depression by Promoting GSK-3β Phosphorylation to Enhance the Wnt/β-catenin Activation in the Hippocampus of Rats. *J Explor Res Pharmacol* 2021;6(4):148–157. doi: 10.14218/JERP.2021.00016.

Introduction

Depression is a chronic mental illness that has a high recurrence rate and is commonly accompanied by the clinical symptoms of insomnia, neurasthenia, and even suicide attempts.[1] According to the global burden of disease study, depression might become the leading cause of disability worldwide by 2030.[2,3] Currently, the treatments for depression include selective serotonin reuptake inhibitors, tricyclic antidepressants, which often take weeks to months to achieve antidepressant effects, and have various side effects. Therefore, novel antidepressants need to be developed that are safe and effective and to elucidate their pharmacological mechanisms.

Glycogen synthase kinase-3 (GSK-3) is a ubiquitous serine/threonine protein kinase[4,5] that consists of GSK-3α and GSK-3β. GSK-3β contains four distinct phosphorylation sites, and its serine phosphorylation (Ser21 and Ser9) is associated with suppression of its kinase activity and leads to Wnt/β-catenin activation.[6,7] Recent research into GSK-3β in mental disorders led to significant interest in the antidepressant role of GSK-3β. Wnt ligands can bind to Frizzled receptors and their coreceptors LRP5/6, to phosphorylate Disheveled (Dvl),[8] and then phosphorylate GSK-3β, which leads to the stabilization, accumulation, and nuclear translocation of β-catenin. During this, β-catenin in the nucleus forms complexes with T cell factor/lymphoid enhancer factor to activate their targeted gene expression, which includes the cell-cycle regulator's cyclin D1 and c-myc,[9] and genes involved in synaptic plasticity and memory.[10,11] In addition, GSK-3β phosphorylation can regulate hippocampal neurogenesis and neuroprotection in the CA1 and dentate the gyrus areas of the hippocampus in the brain. This is important in the treatment of depression.[12]

Traditional Chinese medicine offers several options for the treatment of depression that are effective with few toxic side effects.[13,14] The compound Gaoziban tablet (CGZBT) is a Chinese medicine and that can calm the pulse. CGZBT can relieve insomnia, headache, neurasthenia and other clinical symptoms, and has antidepressant effects. However, the mechanisms that underlie the action of CGZBT in depression remain unclear. Therefore, this study evaluated the effect of CGZBT on depression and determined its pharmacological mechanisms in rats.

Materials and methods

Chemicals and reagents

CGZBT was provided by the Xinjiang Uygur Pharmaceutical (Z65020165, Urumqi, China). The main components of CGZBT included *Anchusa azurea* Mill., *Centaurea behen* L., *Santalum album* L., *Limonium gmelinii* (Willd.) Kuntze, *Dracocephalum moldavica* L., *Lepidium sativum* L., *Perilla frutescens* (L.) Britton, *Polygonum japonicum* Meisn., *Lavandula angustifolia* Mill., and *Coriandrum sativum* L and *Silkworm cocoon*. CGZBT tablets were powdered and dissolved in deionized water at 1.6 g/mL and stored before use. Fluoxetine (Flu) hydrochloride was purchased from Changzhou Siyao Pharmaceuticals (7686A; Suzhou, China).

Animals and treatments

Male Sprague-Dawley rats (SPF) 160–180 g were obtained from the Hubei Provincial Center for Disease Control and Prevention. All rats were fed a uniform meal and were allowed free access to water under standard laboratory conditions. The rats were maintained at 24 ± 1°C and humidity of 50 ± 5%. After acclimatization for 7 days, the rats were induced for depression by chronic unpredictable mild stress (CUMS), as reported by Willner and Moreau[15,16] with some modifications. These rats were subjected to the following stressors: (1) water deprivation for 24 h; (2) food deprivation for 24 h; (3) oscillation for 10 m; (4) inversion of the light/dark cycle; (5) damp sawdust for bedding (100 g of sawdust bedding in 200 mL of water); (6) tail pinch for 1 m 1 cm from the base of the tail; and (7) forced swimming in ice water for 5 m daily for 42 days in random order. The rats were trained to consume 1% (w/v) sucrose solution for screening. The rats were randomized into the control, CUMS, CUMS + 0.4 g/kg CGZBT, CUMS + 0.8 g/kg CGZBT, CUMS + 1.6 g/kg CGZBT, and CUMS + 10 mg/kg Flu groups (n = 10 per group), respectively. On day 28 post CUMS, the rats were administered CGZBT and Flu by gavage daily throughout the experiment. The experimental protocols were approved by the Animal Ethics Committee of Hubei Center for Disease Control and Prevention (2017-0067).

High-performance liquid chromatography

Compounds in CGZBT were identified using high-performance liquid chromatography (HPLC) using the standard substances as the controls. Standard substances included: (1) sinapic acid; (2) rutin; (3) rosmarinic acid; (4) myricetin; (5) quercetin; (6) luteolin; (7) kaempferol; and (8) apigenin; (Chengdu Pusi Biotechnology).

The HPLC used an SB-C18 column (4.6 mm × 250 mm, 5 m) by ZORBAX [column temperature maintained at 30°C, sample chamber temperature was 4°C, the injection volume was 10 μL, the detection wavelength was 330 nm, and the flow rate was 1 mL/min. CGZBT was separated by a mobile phase that contained 0.05% phosphoric acid water-methanol)]. The process was (0–5 m, 5%A; 5–17 m, 5%→10%A; 17–20 m, 10%→18%A; 20–25 m, 18%→25%A; 25–35 m, 25%→35%A; 35–40 m, 35%→70%A; 40–45 m, 70%→5%A).

Behavioral evaluation

Anhedonia, which is a central manifestation of depression in humans, was identified using a sugar preference test.[17] Briefly, the rats fasted for 24 h and were provided with two identical bottles that contained water or 1% sucrose solution for 1 h. The locations of these two bottles were randomly swapped to avoid a position preference. Sucrose preference % = sucrose intake/(sucrose intake + water intake) %.

The forced swimming test was developed by Bourin[18] as a method to assess the clinical efficacy of antidepressants in rodents.[19] The original experiment was modified by placing the rats in an open cylindrical white plastic container (80 cm height × 18 cm diameter), which was filled to a height of 30 cm with fresh tap water at 22–25°C. The rats had to swim for 6 m, and the final 4 m was measured for immobility, which was defined as the smallest distance of movement that maintained the rat's head above the water level.

The open field test was performed to evaluate autonomous behavior, exploratory activity, and anxiety in the experimental animals.[20] The experiment used a 40 × 40 × 50 cm^3 test box, the bottom of which was divided into four squares. An automatic video tracking system was used to record total movement, distance, and number of times that the rats crossed the center of the box within 5 m in novel environments.[20]

Serum cytokine levels

Blood samples were obtained from the abdominal aorta of rats and prepared for serum samples by centrifugation. The levels of serum tumor necrosis factor-alpha (TNF-α), interleukin (IL) 1β, IL6, IL4, and IL10 were measured using enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology), according to the manufacturer's instructions (Shanghai Fusheng Industrial). Absorbance was measured at 450 nm in a Spark 10M microplate reader (Tecan, Männedorf, Switzerland).

Measurement of neurotransmitter levels

The animals were euthanized at the end of the experiment. Their hippocampus tissues were dissected and stored at −80°C. The tissues were homogenized and then centrifuged, the norepinephrine (NE), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) levels in individual tissue samples were determined using liquid chromatography-mass spectrometry.[21]

Histological analysis

Fresh hippocampal tissues were fixed in 4% paraformaldehyde for ≥24 h and embedded in paraffin. The tissue sections (5 μm) were stained with hematoxylin-eosin (H&E). The number of CA1 cells in each hippocampus was quantified using the ImageJ V1.51 software in a blinded manner.

Western blot analysis

The fresh hippocampal tissues were homogenized in lysis buffer (RIPA) that contained phenylmethylsulfonyl fluoride (PMSF) protease inhibitors (Beyotime, Shanghai, China). After being centrifuged, the protein concentrations were quantified. The tissue lysate samples (50 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked with 5 % skimmed milk in TBST and probed overnight at 4°C with rabbit anti-Wnt1 (1:1,000 dilution), rabbit anti-β-catenin (1:1,000 dilution), and mouse anti-p-GSK-3β (ser9) (1:1,000 dilution) (Proteintech, USA). After washing, the bound antibodies were reacted with horseradish peroxidase-conjugated goat antimouse/rabbit secondary antibodies (1:2,000 dilution, Proteintech). The immunocomplex was visualized using ECL (Thermo). The relative levels of target protein expression were quantified by densitometric scanning using the FluorChem FC3 system (ProteinSimple, USA).

Immunohistochemistry analysis

The levels of p-GSK-3β (ser9), Axin, adenomatous polyposis coli (APC), Wnt1, and β-catenin in rat hippocampus were examined using immunohistochemistry. The paraffin-embedded hippocampus tissue sections (5 μm) were dewaxed, rehydrated, and treated with 3% H_2O_2 in methanol. The sections were blocked with 5% bovine serum albumin in PBST and probed with rabbit anti-p-GSK-3β (ser9) (1:300, CST, USA), goat anti-α-Axin (1:200), mouse anti-α-APC (1:150), rabbit anti-α-Wnt1 (1:200), and rabbit anti-β-catenin (1:300, Abcam, USA) overnight at 4°C. Then, the sections were incubated with the appropriate biotinylated secondary antibodies (1:200) and then with HRP-conjugated streptavidin. Finally, the sections were stained with DAB and examined under a light microscope.

Statistical analysis

SPSS version 22.0 was used for all statistical analyses (IBM, USA). One-way analysis of variance and a Student's t-test were used to compare the difference between or among groups. Data are expressed as mean ± SD. Statistical significance was set at a p-value <0.05.

Results

HPLC analysis of CGZBT

The major chemical components in CGZBT extracts were analyzed by HPLC (Fig. 1a) using the reference mixture (Fig. 1b). There were eight major components in the extracts, according to the standard substances (Fig. 1c). The analysis of the spectra indicated that these components included sinapic acid, rutin, rosmarinic acid, myricetin, quercetin, luteolin, kaempferol, and apigenin.

CGZBT ameliorates depression-like behaviors in CUMS rats

Following treatment with different doses of CGZBT in the CUMS rats, the animal depression-like behaviors were analyzed. There was no significant difference in sucrose preference, immobility time, and distance across the center between six groups in the first behavioral test (Figs. 2b–d, x = 1). After drug therapy for 2 weeks, treatment with CGZBT at 0.4 g/kg or a higher dose significantly improved sucrose preference, and reduced immobility time (both p<0.01, Figs. 2b and c, x = 3). Compared with the control group, the rats in the CUMS group demonstrated significantly decreased activity (p<0.01). This reduction in activity was alleviated via CGZBT treatment (0.4, 0.8, 1.6 g/kg, all p<0.01). Treatment with CGZBT increased movement, traveling distance, and the number of center crossings (Figs. 2d and e). Therefore, CGZBT treatment ameliorated depression and anxiety-like behaviors in the CUMS rats.

CGZBT regulates the levels of NE, 5-HT, and 5-HIAA in the hippocampus in CUMS rats

Then, the levels of neurotransmitters, such as NE, 5-HT, and 5-HIAA in the hippocampus of the rats were measured by liquid chromatography-mass spectrometry. Compared with the control group, the levels of NE, 5-HT, and 5-HIAA in the hippocampus of the CUMS group were significantly reduced (Figs. 3a–c, p<0.01), which suggested potential brain injury. Of note, treatment with CGZBT at 0.4, 0.8, 1.6 g/kg increased the levels of NE, 5-HT, and 5-HIAA in the hippocampus of rats. In particular, treatment with CGZBT significantly increased the levels of NE in rats (p<0.01).

CGZBT alters the number of hippocampal CA1 cells in CUMS rats

The impact of CGZBT treatment on the morphological changes

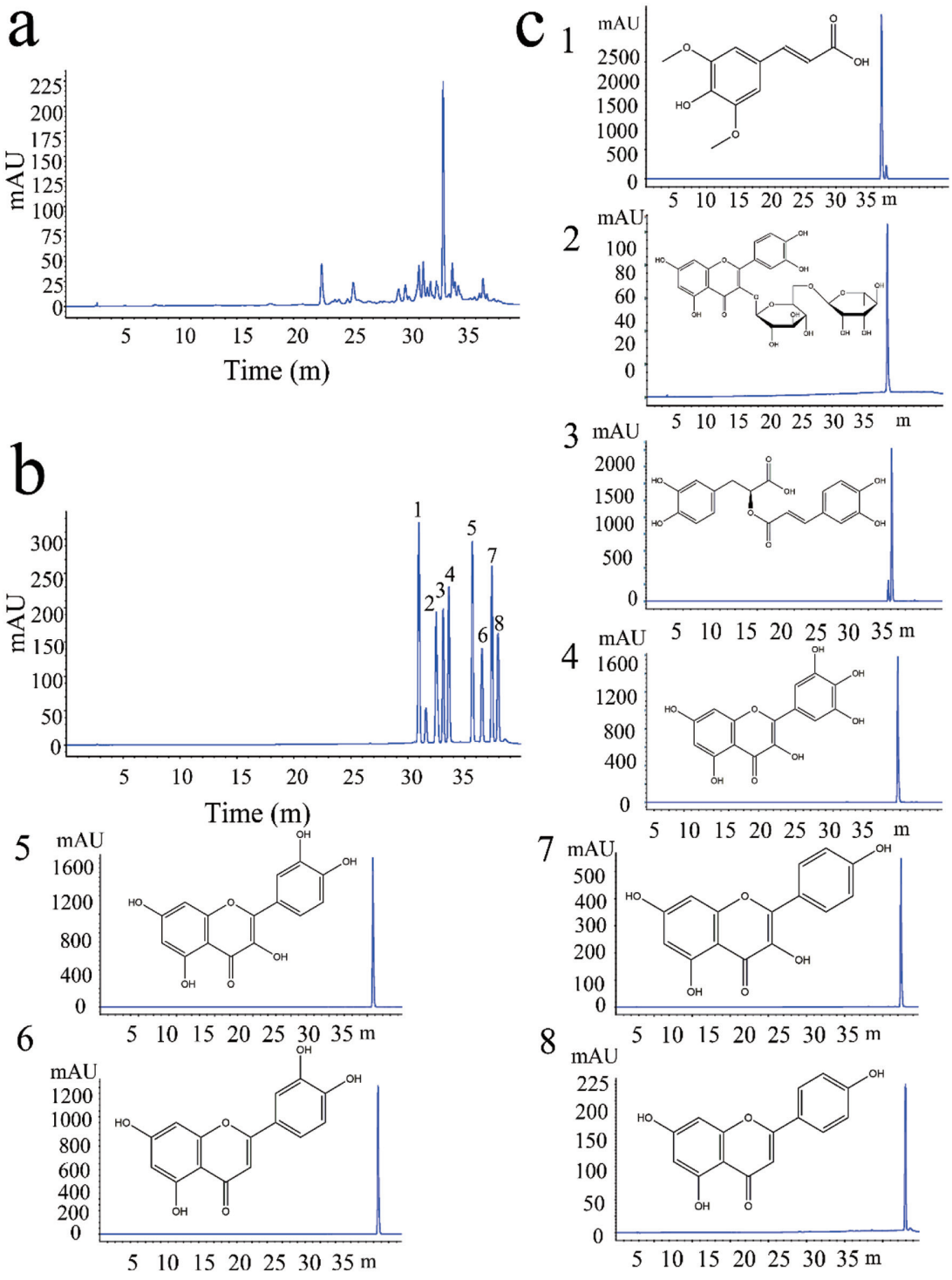

Fig. 1. HPLC analysis of CGZBT. (a) chromatogram of CGZBT; (b) chromatogram of the mixture of eight standard substances; and (c) chromatogram of eight individual standard substances, including (1) sinapic acid; (2) rutin; (3) rosmarinic acid; (4) myricetin; (5) quercetin; (6) luteolin; (7) kaempferol; and (8) apigenin. CGZBT, compound gaoziban tablet; HPLC, high-performance liquid chromatography.

in the hippocampal tissues of rats was examined. H&E staining revealed that there was an obvious change in cell morphology in the hippocampal CA1 region of CUMS rats (Fig. 4a). Compared with the control, the number of CA1 cells was significantly reduced in CUMS rats (Fig. 4b; $p<0.01$) and restored by treatment with CGZBT (0.4, 0.8, 1.6 g/kg) treatment ($p<0.01$). Therefore, CGZBT treatment mitigated the CUMS-decreased CA1 cells in the hippocampus of rats.

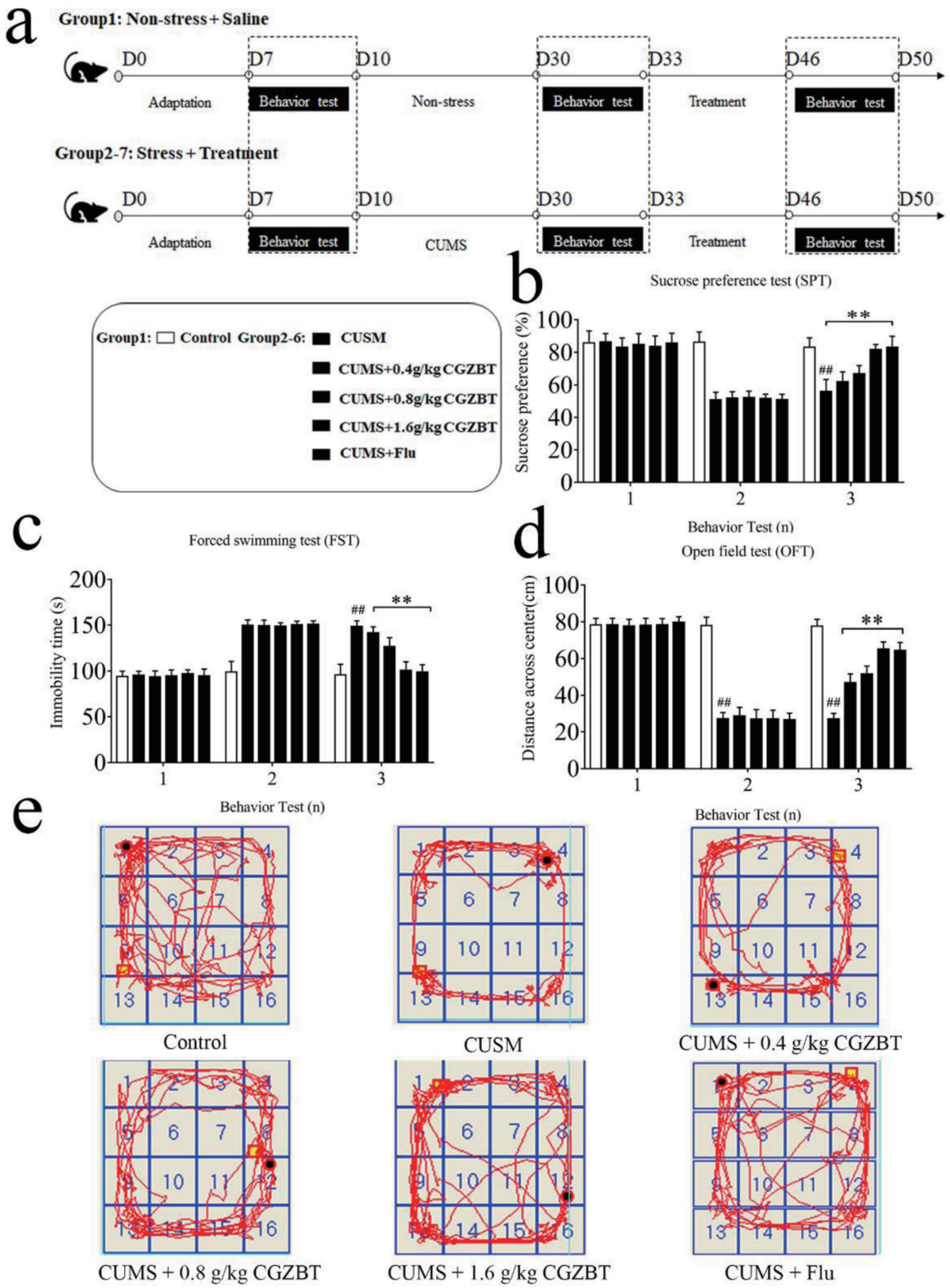

Fig. 2. CGZBT ameliorates depression-like behaviors in CUMS rats. (a) diagram of experimental design; (b–d) depressive-like behaviors were quantified by SPT, FST, and OFT. The *x*-axis in 1, 2, and 3 represent the behavioral tests on days 7, 30, and 46 in Figure a; and (e) charts of tracking movement in rats. Data are expressed as mean ± SD. $^{\#\#}p$ 0.01 compared with the control group. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the CUMS group. CGZBT, compound gaoziban tablet; CUMS, chronic unpredictable mild stress; FST, forced swimming test; OFT, open field test; SPT, sucrose preference test.

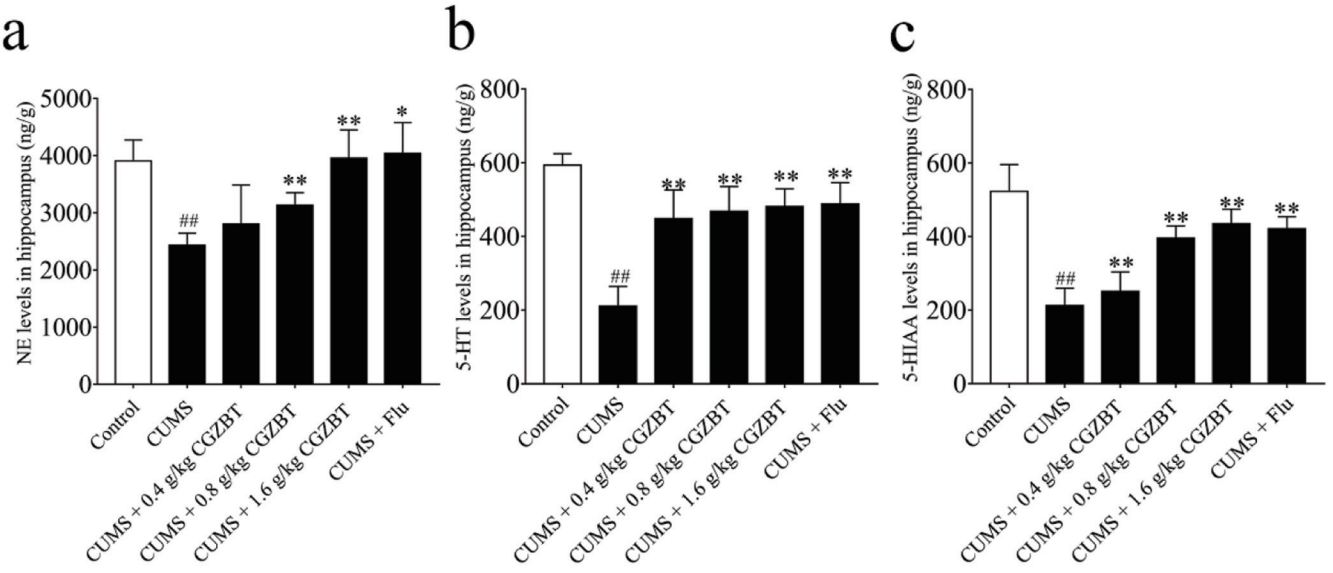

Fig. 3. CGZBT restores the levels of neurotransmitters in the hippocampus. (a–c) levels of NE, 5-HT, and 5-HIAA in individual rats measured by LC-MS. Data are expressed as mean ± SD.$^{\#\#}p<0.01$ compared with the control group. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the CUMS group. 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; CGZBT, compound gaoziban tablet; CUMS, chronic unpredictable mild stress; LC-MS, liquid chromatography-mass spectrometry; NE, norepinephrine.

CGZBT suppresses the GSK-3β mediated Wnt/β-catenin pathway

To further understand the potential mechanism of CGZBT in depression, the expression of GSK-3β regulated Wnt/β-catenin pathway-associated proteins was investigated. Immunohistochemical staining and western blot analysis indicated that the expression of Wnt1, β-catenin, and p-GSK-3β (ser9) significantly decreased in CUMS rats, relative to the control group (Figs. 5a–c; $p<0.01$). Of note, this change was mitigated after treatment with CGZBT or Flu ($p<0.05$ and $p<0.01$, respectively). Immunohistochemical staining revealed that the expression of Axin and APC increased in the CUMS group compared with the control group (Fig. 5a). Treatment with CGZBT mitigated the changes. Treatment with CGZBT at 0.4, 0.8, and 1.6 g/kg significantly decreased the concentrations of serum TNF-α, IL1β, and IL6 (Figs. 5d–h; $p<0.01$). Of note, administration with CGZBT (1.6 g/kg) was more effective at inhibiting serum IL6 levels. In contrast, the levels of serum IL4 and IL10 in CUMS rats were significantly lower than that in the control and were elevated by treatment with 1.6 g/kg CGZBT in CUMS rats.

Discussion

In this study, CGZBT treatment increased neurotransmitter levels, improved the number of cells in the hippocampus CA1 region, inhibited inflammatory cytokines, and enhanced the activation of Wnt/β-catenin signaling by promoting GSK-3β phosphorylating in CUMS rats. In combination, these effects improved depression in CUMS-induced rats.

CGZBT has been mainly used for the treatment of neurasthenia, dizziness, and other ailments because it has effects on cardiac tonic and protecting the brain. This study identified that rutin myricetin, quercetin, luteolin, kaempferol, and apigenin were the major components of CGZBT and these components were flavonoids. Research has shown that flavonoids have anti-inflammatory,[22] neuroprotective,[23] immunomodulatory, and antidepressant effects.[24] However, how these components interact and affect depression need to be investigated.

CUMS is a well-established animal model of depression that is induced by CUMS.[25] This model is a useful animal model to evaluate antidepressant activity.[26] The behavioral changes in rats are similar to individuals with depression and can be reversed by chronic rather than acute treatment with antidepressants, as in the clinical situation.[27,28] Continual administration with CGZBT for 14 days effectively mitigated the depressive-like behaviors in CUMS rats. Therefore, this study provided experimental evidence for the antidepressant efficacy of CGZBT.

Depression is linked to alterations in the levels of neurotransmitters within the brain.[29] Previous studies showed that serotonergic and norepinephrine receptors are involved in depression-like effects by regulating concentrations of NE and 5-HT within the synaptic cleft.[30] In this study, treatment with CGZBT restored the levels of NE, 5-HT, and 5-HIAA in the hippocampal tissues of CUMS rats, which could contribute to their antidepressant effect by enhancing the monoamine neuronal system.

Emerging evidence reveals that neuronal injury is an important factor in the pathogenesis of depression, and is characterized by neuronal injury and cell death (mainly in the form of necrosis and apoptosis) in specific brain regions.[31,32] Various studies, which include neuroimaging and autopsy studies, have shown that depressed patients have decreased neurons in the brain.[33] In this study, a reduction in the cell numbers in the hippocampal CA1 region was observed in the CUMS rats, CGZBT treatment significantly improved the number of cells in the hippocampal CA1 regions of CUMS rats. This suggested that CGZBT treatment might protect the CA1 neurons from death through a pleiotropic mechanism.

Previous studies validated the role of GSK-3β in the pathogenesis of mood disorders.[6,34,35] In addition, GSK-3β inhibitors have an antidepressant effect by reducing inflammation, promoting neurogenesis, and regulating the homeostasis of 5-HT.[36,37] OF interest, in autopsies of patients with major depressive disorders, in-

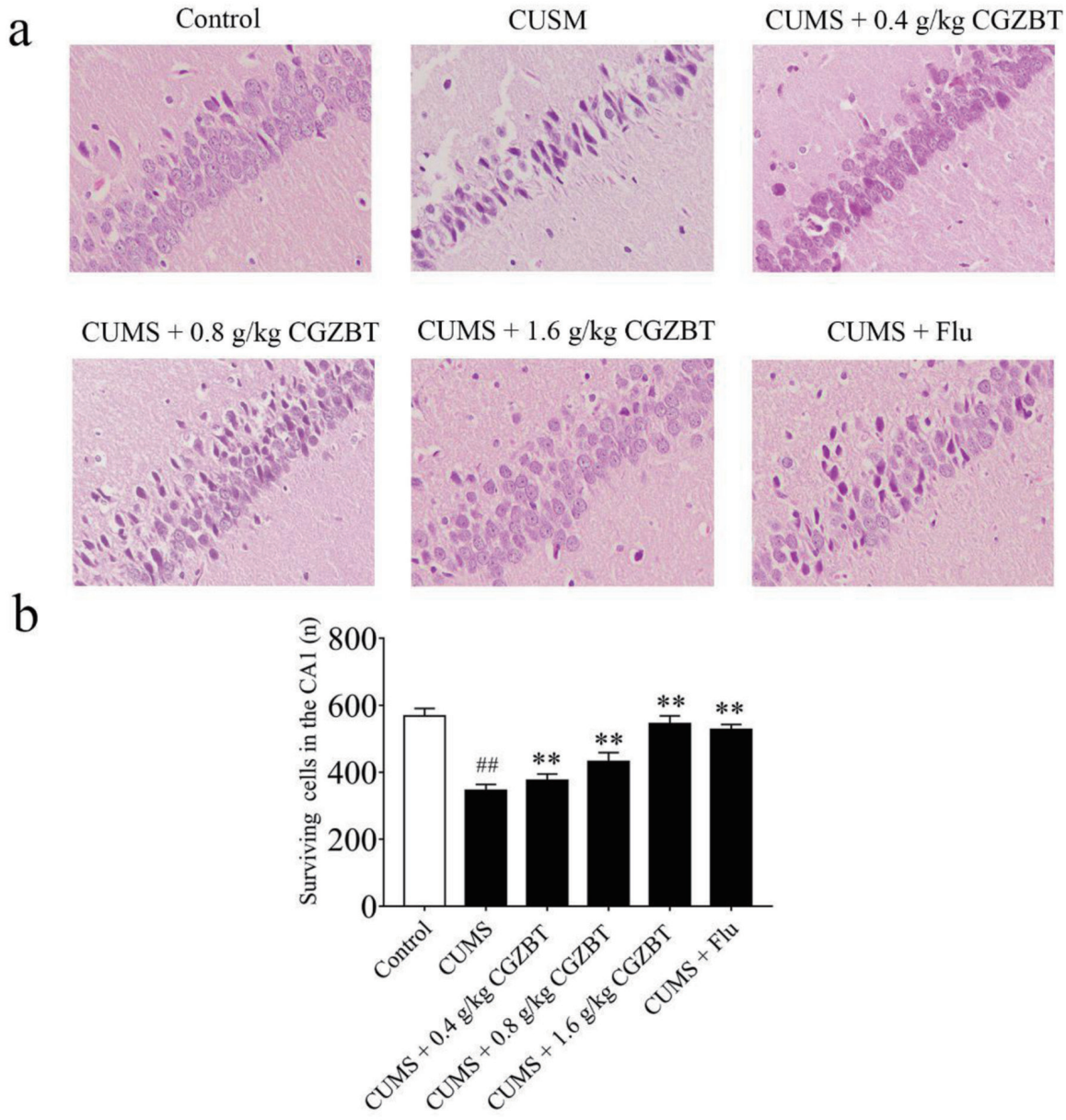

Fig. 4. CGZBT alters the number and morphology of hippocampal CA1 cells. (a) representative micrographs displayed the morphology and distribution of CA1 cells; and (b) histograms of the number of cells in the CA1 region (200 × magnification). Data are expressed as mean ± SD. $^{\#\#}p<0.01$ compared with the control group. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the CUMS group. CGZBT, compound gaoziban tablet; CUMS.

creased GSK-3β activity and decreased β-catenin expression were observed in their brain.[4,38,39] In this study, the levels of β-catenin and p-GSK-3β (ser9) decreased in CUMS rats, which suggested that during the pathogenic process of depression, strong GSK-3β activity reduces the stability of β-catenin in the brain of CUMS rats. Therefore, the hippocampal β-catenin level might be a marker to evaluate the antidepressant activity of GSK-3β inhibitory drugs. In addition, the western blot results showed that CGZBT treatment restored the levels of Wnt1, β-catenin, and p-GSK-3β (ser9) in the hippocampal tissues, and reduced Axin and APC expression. Axin forms a complex with APC and GSK-3β to promote β-catenin degradation, which is a key regulatory step in Wnt/β-catenin signaling.[40] Aberrant GSK-3β activity can induce inflammation in the peripheral and the central nervous system[41] because it can activate different types of toll-like receptors.[42] However, inhibition of GSK-3β can promote nf-kappaB (NF-κB) activation, and reduce cyclic BP/P300 protein binding to the cyclic AMP response element binding protein,[43] which can increase IL-10 expression. In this study, the administration with CGZBT reduced the levels of serum TNF-α, IL-1β, and IL-6 and increased IL-4 and IL-10 levels in CUMS rats. In combination, these results indicated that the therapeutic antidepressant effect of CGZBT might be mediated by increasing GSK-3β phosphorylation to enhance Wnt/β-catenin signaling that protects the CA1 neurons in the hippocampus and reduces systemic inflammation in rats. Therefore, our findings might provide insights into the pharmacological mechanisms that

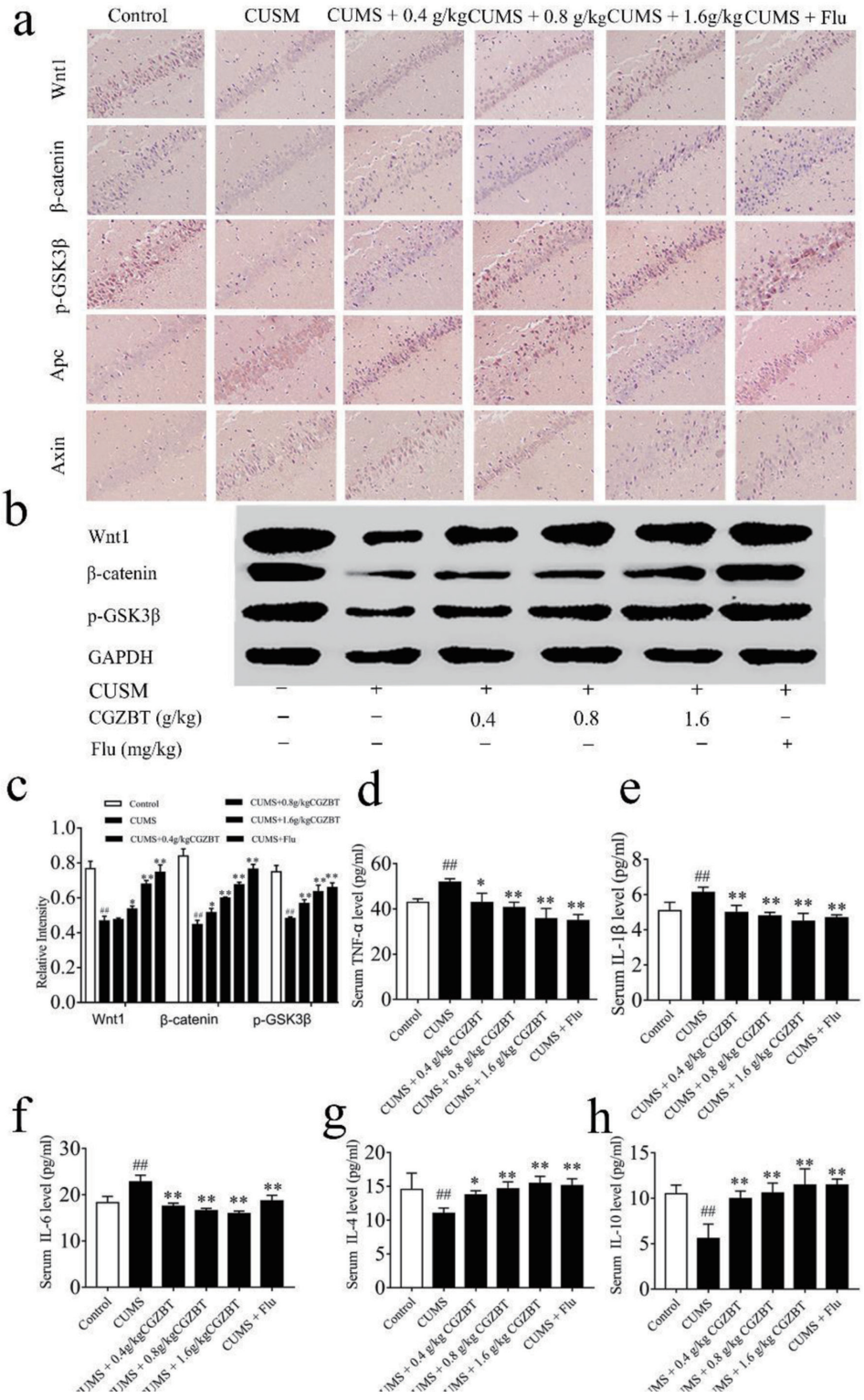

Fig. 5. CGZBT suppresses the GSK-3β activity to enhance the Wnt/β-catenin signaling activation and inhibits inflammation in the hippocampal tissues of CUMS rats. (a) immunohistochemical staining of Wnt1, β-catenin, p-GSK3β (ser9), APC, and Axin expression in the hippocampus (200 × magnification); (b) western blot analysis of the relative levels of Wnt1, β-catenin, and p-GSK-3β (ser9) expression in the hippocampus; (c) quantitative analysis of western blot data. CGZBT attenuates CUMS-induced inflammation; and (d–h) levels of serum TNF-α, IL1β, IL6, IL4, and IL10 were quantified by ELISA. Data are expressed as mean ± SD. $^{\#\#}p<0.01$ compared with the control group. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the CUMS group. CGZBT, compound gaoziban tablet; CUMS, chronic unpredictable mild stress; ELISA, enzyme-linked immunosorbent assay; GSK-3β, glycogen synthase kinase-3β; TNF-α, tumor necrosis factor-alpha.

underlie the action of CGZBT in antidepressant activity and aid the design of therapies for depression in the clinic.

Future directions

In a rat model of CUMS, CGZBT improved depression-related behaviors. The compounds identified in the CGZBT extracts were flavonoids. Therefore, further analysis of the antidepressant effect of CGZBT extracts is required, which could be a basis for the development of new antidepressant agents.

Conclusions

The results indicated that CGZBT could effectively improve depression. In combination, our findings demonstrated that the effect of CGZBT in depression is related to promoting GSK-3β phosphorylation to enhance Wnt/β-catenin activation in the hippocampus of rats. Our findings suggest that GSK-3β might be a common signaling event for the convergence of different psychotropic medicines and provides novel targets for the development of new antidepressant drugs.

Acknowledgments

None.

Funding

This work was supported by the Hubei Provincial Natural Science Foundation of China (No.2018CFB657).

Conflict of interest

Hailong Yin and Qiang Yin are the employees of Xinjiang Uygur Pharmaceutical (Urumqi, China).

Author contributions

Study design (XSZ, HLY and LS); statistical analysis (HPL, MHW and WCS); protocol writing (YL, WLC); first draft of the manuscript (XSZ); study analysis and final manuscript editing (HZW, YFY, JFZ, YWL, HXD, QY and PTY); literature searches (XSZ, LS).

Data sharing statement

Data from this study can be obtained from the authors upon request.

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