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ORIGINAL RESEARCH

Antidepressant-Like Effect and Mechanism of Ginsenoside Rd on Rodent Models of Depression

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Background: There is growing evidence to suggest that ginsenoside Rd (GRd) has a therapeutic effect on depression, but the specific mechanisms behind its activity require further study.

Objective: This study is designed to investigate the antidepressant-like effect and underlying mechanisms of GRd.

Methods: In this study, the behavioral despair mouse model of depression and chronic unpredictable mild stress (CUMS) rat model of depression were established to explore the effects of GRd on depression-like behavior and its underlying mechanisms. Behavioral tests were used to evaluate the replication of animal models and depression-like behaviors. The hypoxia-inducible factor-1α (HIF-1α) blocker 2-methoxyestradiol (2-ME) was injected to determine the role of HIF-1α in the antidepressant-like effect of GRd. In addition, molecular biology techniques were used to determine the mRNA and protein expression of HIF-1α signaling pathway and synaptic plasticity-related regulators, that is synapsin 1 (SYN 1) and postsynaptic density protein 95 (PSD 95). In silico binding interaction studies of GRd with focused target proteins were performed using molecular docking to predict the affinity and optimal binding mode between ligands and receptors.

Results: Our data show that GRd significantly reversed depression-like behavior and promoted mRNA and protein expression of HIF-1α signaling pathway and synaptic plasticity-related regulators. However, the antidepressant-like effect of GRd disappeared upon inhibition of HIF-1α expression following administration of 2-ME. Furthermore, molecular docking results showed that GRd possessed significant binding affinity for HIF-1α, VEGF, and VEGFR-2.

Conclusion: Our results show that GRd exhibits significant antidepressant-like effect and that HIF-1α signaling pathway is a promising target for the treatment of depression.

Keywords: ginsenoside Rd, antidepressant effect, HIF-1α-VEGF signaling pathway, VEGFR-2, synaptic plasticity-related regulators, molecular docking

Introduction

Depression is a mental affective disorder characterized by significant and long-time decreased interest, low mood, slow thinking, activity decline, and loss of appetite, which seriously affects the physical and mental health of patients.[1] According to a report from the World Health Organization, depression is predicted to become the main cause of the global burden of disease by 2030.[2] At present, the treatment strategy for depression still involves selective serotonin (5-HT) reuptake inhibitors (SSRIs) which have been traditionally used in the clinic. Unfortunately, SSRIs do not meet the criteria for an excellent therapy, including rapid onset of action, high curative rate, and fewer side effects. SSRIs are ineffective in up to 35% of patients and they cause many side effects, such as gastrointestinal adverse reactions (particularly nausea and vomiting) and neurological side effects (especially headaches and tremors).[3–5] Moreover, depression has become a serious worldwide public health problem and its complicated pathological mechanisms need

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to be further explored. Consequently, addressing this unmet need requires further study to develop safe and effective antidepressant drugs and explore innovative mechanisms of action.

Panax ginseng Meyer, a precious traditional Chinese medicine, is indigenous to the Far East countries (most notably China and Korea).[6] According to the Pharmacopoeia of the People's Republic of China (2020), it has the effect of reinforcing vital energy, calming the mind, and boosting cognitive functions. Further, antidepressant was one of the clinical indications of ginseng according to traditional Chinese medicine, with ginseng extract also being reported to improve depression in postmenopausal women.[7,8] Modern research has proven that ginsenosides are the active ingredients of ginseng, traditionally used to treat insomnia, heart palpitations, mood disorders, and depression.[9–11] Kaixin San has been used clinically for the prevention and treatment of depression, and many studies have shown that ginsenosides are most likely its main medicinal ingredients.[12,13] In rodent depression models, ginseng total saponins and monomers, including but not limited to, Rb1, Rg1, and Rg3 have shown great antidepressant activity.[14–17] These studies support the hypothesis that ginsenosides may be promising antidepressant drugs.

Naturally occurring ginsenosides have poor oral availability. Researchers generally believe that ginsenosides act as prodrugs in pharmacology when taken orally, and that their metabolites may be responsible for their observed efficacy due to their improved pharmacological activity.[18] Ginsenoside Rd (GRd), which is produced by the metabolism of the hydrophilic protopanaxadiol ginsenoside via intestinal microbiota, is one of the most active and abundant components involved in the efficacy of ginseng. GRd has a variety of pharmacological activities, especially in terms of neuroprotection and shows improved efficacy compared to other ginsenosides.[19] The potential mechanisms of GRd neuroprotection may be related to anti-inflammation, antioxidant, anti-apoptosis, and the regulation of Ca^{2+} and nerve factors by modulation of NF-κB, PI3K, and MAPK signaling pathway.[20] Recent studies have demonstrated that GRd could improve cognitive impairment caused by chronic stress and play a great antidepressant effect in rodent models of depression.[21,22] However, the mechanism by which GRd improves depression is not clear, thereby necessitating further investigation.

Hypoxia-inducible factor-1 (HIF-1), a heterodimer composed of HIF-1α and HIF-1β, plays an important role in the process of cells perceiving and adapting to changes in oxygen partial pressure in the internal environment. As the regulatory subunit of HIF-1, HIF-1α regulates HIF-1 activity through its sensitivity to changes in oxygen concentration.[23] There is increasing evidence to suggest that HIF-1α may be a new target to treat depression. Studies have shown that intermittent hypoxia could promote hippocampal neurogenesis in adult rats and exert antidepressant-like effects on a variety of animal models for screening antidepressant activity, which is closely related to the activation of HIF-1α.[24] The activation of HIF-1α may mediate synaptic plasticity to exert an antidepressant-like effect, which can be completely blocked when HIF-1α synthesis is inhibited by lentiviral infection with HIF-1α small hairpin RNAs.[25] HIF-1α targeting genes vascular endothelial growth factor (VEGF) and erythropoietin (EPO) also have similar effects. VEGF is essential for inducing the rapid antidepressant-like effects, neurotrophic actions, and synaptic effects of ketamine. Peripheral administration of EPO can also produce a strong antidepressant effect. These studies suggest that HIF-1α is a promising therapeutic target for depression.[26,27]

Many studies have shown that GRd acts on the HIF-1α-VEGF pathway to play multiple positive roles. These include pro-angiogenesis after ischemic stroke and neurogenesis after transient focal cerebral ischemia.[28,29] This suggests that GRd may also achieve a therapeutic effect in depression through this signaling pathway. To verify this hypothesis, the behavioral despair mouse model of depression and the chronic unpredictable mild stress (CUMS) rat model of depression were established to explore the antidepressant-like effect and specific mechanisms of GRd. Moreover, GRd was selected as the ligand for focused docking into HIF-1α, VEGF, and VEGFR-2 for molecular docking studies to evaluate protein-ligand binding and to further validate GRd as a potential ligand in other targets for therapeutic intervention in disease.

Materials and Methods

Animals and Drugs

Experiments were performed on adult male ICR mice (SPF grade, 18–22 g) and male Sprague Dawley rats (SPF grade, 180–220 g). All animals were obtained from SiPeiFu (Beijing) Biotechnology Co., Ltd., animal license number: SCXK (Beijing) 2019-0010. Before the actual experiments, animals were allowed one week of adaptive feeding. In addition, all

animals were kept in the following standard conditions, including a 12/12 h light/dark cycle (light on 8:00 a.m. and light off 8:00 p.m.), at ambient temperature (21–23°C), humidity range of 30–50%, and adequate standard food and water. All steps involving animals were authorized by the Experimental Animal Ethics Committee of the Academic Committee of Beijing University of Chinese Medicine (project identification code: BUCM-2021082802-3002). The experimental procedures of our study were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

GRd and paroxetine were obtained from Yuanye (purity >98%, Shanghai, China) and 2-Methoxyestradiol (2-ME) was obtained from APE×BIO (Houston, USA).

Animal Study Design

Behavioral Despair Mouse Model of Depression

Forty mice were randomly divided into four groups: control (Con), paroxetine (Par, 10 mg/kg, i.p.), low-dose GRd (L-GRd, 10 mg/kg, i.g.), and high-dose GRd (H-GRd, 20 mg/kg, i.g.). All drugs were administered once a day for 14 days, while mice in the Con group received an equal volume of saline. Behavioral tests were performed on day 14. All mice were intraperitoneally anesthetized with 1% pentobarbital. The hippocampus of each mouse was removed and stored on ice for analysis.

HIF-1α Inhibition

Forty mice were randomly divided into four groups: control (Con), 2-Methoxyestradiol (2-ME), high-dose GRd (H-GRd) and H-GRd+2-ME. Mice in the H-GRd group were intragastrically treated with GRd (20 mg/kg) once daily for 14 days. The GRd dose used was optimal dose identified in Behavioral Despair Mouse Model of Depression. Mice in the GRd +2-ME group were intraperitoneally injected with 2-ME (5 mg/kg) after intragastric administration of GRd. The Con group received equal volumes of saline and mice in the 2-ME group were intraperitoneally injected with 2-ME (5 mg/kg) after receiving saline. Behavioral tests were performed on day 14.

CUMS Rat Model of Depression

Sixty rats were randomly divided into six groups: control (Con), model (CUMS, CUMS+saline), CUMS+Par (CUMS +4.8 mg/kg paroxetine, i.p.), CUMS+L-GRd (CUMS+10 mg/kg GRd, i.g.), CUMS+H-GRd (CUMS+20 mg/kg GRd, i.g.), and H-GRd (20 mg/kg GRd, i.g.). All rats, except those in the Con and H-GRd groups, were exposed to unpredictable mild stressors for 28 days. Subsequently, each group of rats received corresponding drug for 21 days. The behavioral tests including open field tests (OFTs) and sucrose preference tests (SPTs) were performed on day 21 of dosing. All rats were anesthetized by intraperitoneal injection of 1% pentobarbital and the hippocampus of each rat was removed and stored on ice for analysis.

CUMS Protocol

With the exception of the Con and H-GRd groups, rats were exposed to one or two of the following stressors accompanied by single cage breeding for 28 days: deprivation of food and water, reversal of day and night, cage tilt, 4°C ice water swimming, noise, stroboscopic, wet bedding in cages, body restraint, horizontal shaking, and tail pinching. The same stressor was not repeated within a 7 day period to prevent the rats from predicting upcoming stressors.

Forced Swimming Tests

Mice were taken to the testing room to adapt for 1 h before forced swimming tests (FSTs). A single mouse was placed in a cylinder-shaped glass container (height: 25 cm, diameter: 10 cm) filled with tap water (water height: 10 cm, temperature: 24±1°C). When mice were swimming, their tails and limbs could not reach the bottom of container while the tip of their nose was above water to breathe. Containers were separated by an opaque plate to prevent two mice from observing each other. After 2 min of adaptive swimming, the cumulative immobility time of mouse within 4 min was automatically collected and analyzed by behavior analysis system (Etho-Vision XT9, Noldus, Netherlands). The standard criterion for determining the immobility of mice was as follows: mouse stopped struggling and floated on the water, or made only minor movements necessary to keep its head above the water.

OFTs

Animals were taken to the testing room to adapt for 1 h before OFTs. The tests used an opaque square autonomous activity box (mice: 45 cm × 45 cm × 30 cm; rats: 100 cm × 100 cm × 40 cm). The bottom of the box was divided into twenty-five identical squares with nine squares in the middle set as the central area. A single mouse or rat was placed in the center of the autonomous activity box and the behavior analysis system described in Forced Swimming Tests was used to record the movement distance, velocity, frequency of entering the center, and the duration of the stay in the center within 5 min. The environment was kept quiet with stable light during the tests. Before each experiment, the area was cleaned with alcohol to remove all feces and to remove any odor left by previous mice.

SPTs

The SPT is a classic method to assess anhedonia in animals. Briefly, rats were individually placed in cages equipped with two bottles of 1% (w/v) sucrose solution for adaptation training for 24 h. Subsequently, one of the bottles of sucrose solution was replaced with tap water for 24 h. During the third 24 h period, rats were deprived of food and water. Immediately after the third phase, rats had free access to two bottles of solution in the cage for 2 h, one bottle filled with 100 mL of 1% sucrose solution and the other bottle containing 100 mL of tap water. The positions of the two bottles were alternated every hour to prevent positional preference. The consumption of both solutions were recorded and the sucrose preference was calculated as the sucrose preference rate (%): sucrose consumption/(sucrose consumption + tap water consumption) × 100.

Real-Time Fluorescence Quantitative Polymerase Chain Reaction Analysis

To detect mRNA expression of relevant genes, total RNA was first extracted from hippocampal tissue using the Hipure Total RNA Mini Kit (MAGEN) according to the manufacturers instructions. An ultraviolet spectrophotometer (UV-2000, Unico) was used to measure RNA concentration of each sample and RNA samples were maintained on ice to prevent degradation. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and the Power SYBR Green PCR Master Mix (Invitrogen) according to the manufacturer's instructions. The following conditions were used on a T100 Thermal Cycler PCR machine (Bio-Rad, USA): 42°C for 1 h, 70°C for 5 min, storage at 4°C. Amplification and quantitative detection were performed in a Real-Time PCR machine (Bio-Rad, USA) using the following protocol: initial denaturation at 95°C for 10 min, denaturation at 95°C for 10s, annealing at 60°C and extension for 30s with a total of 50 amplification cycles. We used the $2^{-\Delta\Delta Ct}$ method to calculate the relative quantitative analysis of the results. All primer sequences are shown in Table 1.

Western Blotting

Hippocampal tissue was homogenized in RIPA lysis buffer with protease- and phosphatase inhibitors using an electric homogenizer then lysed on ice for 20 min before centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was collected and the pellet was discarded to obtain the whole cell lysates. The total protein concentration was determined according to the instructions of a BCA protein quantification kit. A total of 40 μg of protein was separated by electrophoresis on a 5–8% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a 0.45 μm PVDF membrane by wet transfer at 100 V constant voltage for 1.5 h. Subsequently, the membrane was blocked with 5% (w/v) non-fat powdered milk at room temperature for 2 h. After 3×10 min washes in TBST buffer, the membrane was incubated with primary antibody at 4°C overnight and then further washed 3 times before incubation with secondary antibodies at room temperature for 1 h. Blots were developed using an ECL chemiluminescence detection kit. The labeled proteins were visualized using an Azure multifunctional molecular imaging system (C600, Azure, USA).

The primary antibodies and their dilution ratios were as follows: anti-HIF-1α (1:1000, Abcam), anti-VEGF (1:3000, Proteintech), anti-VEGFR-2 (1:800, Proteintech), anti-t-PI3K (1:10,000, Proteintech), anti-p-PI3K (1:500, Abcam), anti-t-Akt (1:5000, Proteintech), anti-p-AKT (1:5000, Proteintech), anti-t-mTOR (1:25,000, Proteintech), anti-p-mTOR (1:1000, Cell Signaling Technology), anti-SYN 1 (1:6000, Proteintech), anti-PSD 95 (1:3000, Proteintech), and anti-α-Tubulin (1:5000, Proteintech). The secondary antibodies and their dilution ratios were as follows: HRP-conjugated affinipure goat anti-rabbit IgG (1:5000, Proteintech) or HRP-conjugated affinipure goat anti-mouse IgG (1:5000, Proteintech).

Table 1 Primer Sequences Used for RT-PCR

Name of Gene	Primer	Sequence (5′ to 3′)
Mouse HIF-1α	Forward	TAGACTTGGAAATGCTGGCTCCCT
	Reverse	TGGCAGTGACAGTGATGGTAGGTT
Mouse VEGF	Forward	AAGCAGATGGTCAAATCG
	Reverse	GGGGCATTAGAAGGTTGT
Mouse β-Actin	Forward	ACTCCTATGTGGGTGACGAGG
	Reverse	CACACGCAGCTCATTGTAGAAG
Rat HIF-1α	Forward	GTCAGCAACGTGGAAGGTGC
	Reverse	GCACCAAGCACGTCATAGGC
Rat VEGF	Forward	GCAGTGCTCCCCATCCGCTG
	Reverse	TGCTCGTCCGACAGCTGGGA
Rat VEGFR-2	Forward	TGGCAATTCCCGTCCTCAAAGC
	Reverse	CTTGGTCACTCTTGGTCACACTGTC
Rat PI3K	Forward	GCAACTCCTGGACTGCAACT
	Reverse	CAGCGCACTGTCATGGTATG
Rat AKT	Forward	TAGCCATTGTGAAGGAGGGC
	Reverse	CCTGAGGCCGTTCCTTGTAG
Rat mTOR	Forward	GCTCCAGCACTATGTCACCA
	Reverse	CGTCTGAGCTGGAAACCAGT
Rat β-Actin	Forward	CATCCTGCGTCTGGACCTGG
	Reverse	TAATGTCACGCACGATTTCC

Molecular Docking

Three-dimensional (3D) structures of focused target proteins including HIF-1α, VEGF, and VEGFR-2 implicated in depression were obtained from the online RCSB Protein Data Bank (PDB; https://www.rcsb.org/). PyMOL 2.4 (Delano Scientific LLC, Italy) was used to remove water molecules and small molecule ligands from protein structure. AutoDock Tools was then used to add hydrogen and atom types, to calculate charge, and finally for conversion into the PDBQ format. A two-dimensional (2D) structure of the small molecule ligand (ie GRd) was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Chem3D 19.0 software was then used to convert this into a 3D structure and to optimize the mechanical structure to minimize energy. AutoDock Tools was used to detect the root of the ligand and select the rotatable bond. Finally, this was saved in a PDBQT format. Molecular docking experiments were performed in Autodock Vina (PyRx 0.8).

Statistical Analysis

Data are expressed as mean ± standard error mean (SEM), and data with normal distribution and homogeneity of variance were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. The Kruskal–Wallis test was used for data with non-normal distribution. Statistical analysis was performed using SPSS 26 (SPSS, Chicago, Illinois, USA) and GraphPad Prism 8.0 (GraphPad Software Inc, San Diego, CA, USA). A p value < 0.05 was considered to be statistically significant.

Results

Effects of GRd on Behavioral Despair Mouse Model of Depression

Effects of GRd on Immobility Time in FSTs

The FST is still one of the most commonly used methods for screening antidepressants in all animal models. Compared to the Con group, the forced swimming immobility time of H-GRd, D-GRd, and Par groups were significantly shorter (*p < 0.05; **p < 0.01) (Figure 1).

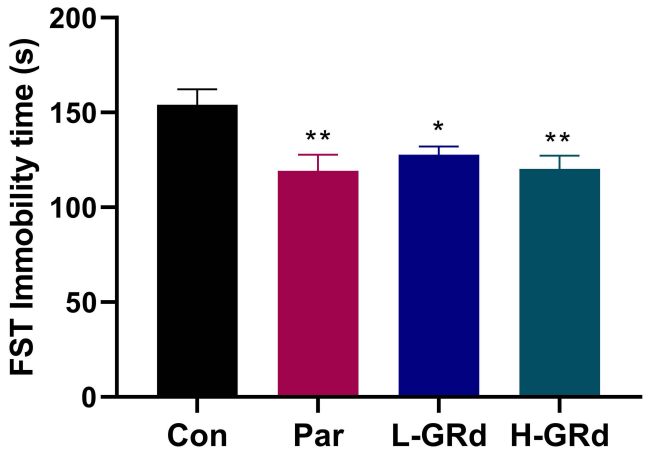

Figure 1 The effects of GRd on immobility time in the FST. Data are expressed as mean ± SEM (n = 10/group). *$p < 0.05$ vs control group; **$p < 0.01$ vs control group.

Effects of GRd on OFTs

OFTs were designed to eliminate the interference of immobile time in the FST. There were no significant differences in movement distance, velocity, frequency of entering the center, and the duration of stay in the center between all groups (Figure 2).

Effects of GRd on HIF-1α-VEGF Signaling Pathway

Changes in the HIF-1α-VEGF signaling pathway were examined in the behavioral despair mouse model of depression to explore potential mechanisms underlying the antidepressant-like effect of GRd. We found that mRNA and protein expression of HIF-1α and VEGF in low and high dose groups of GRd were significantly higher than those in the control group (Figure 3; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Effects of HIF-1α Inhibitor on the Behavioral Despair Mouse Model of Depression

Effects of 2-ME on Immobility Time in the FST

We used 2-ME, a widely used and effective inhibitor of HIF-1α, to block HIF-1α expression to explore its role in the antidepressant-like effect of GRd. Our data show that the forced swimming immobility time of the GRd group was significantly shorter than that of the control group (Figure 4; **$p < 0.01$). This was not the case for the GRd+2-ME group mice. These data suggests that (1) the antidepressant-like effect of GRd can be eliminated by 2-ME, and (2) HIF-1α is essential for this effect. In addition, the forced swimming immobility time of mice treated with 2-ME alone was no different from that of the control group, which ruled out the potential of 2-ME-mediated interference.

Effects of 2-ME on OFTs

There were no significant differences in movement distance, velocity, frequency of entering the center, and the duration of stay in the center between all groups (Figure 5).

Effects of GRd on CUMS Depressive Model in Rats

Effects of GRd on SPT

The SPT is a classic test for anhedonia in animals. The sucrose preference of rats in the CUMS group was significantly lower than that of control group (Figure 6; $^{###}p < 0.001$). In comparison, the sucrose preference of rats following treatment with low and high doses of GRd was significantly higher than the CUMS group (Figure 6; *$p < 0.05$, **$p < 0.01$). There were no differences in sucrose preference between the GRd and paroxetine groups. These results indicate that GRd can reverse CUMS-induced anhedonia in rats.

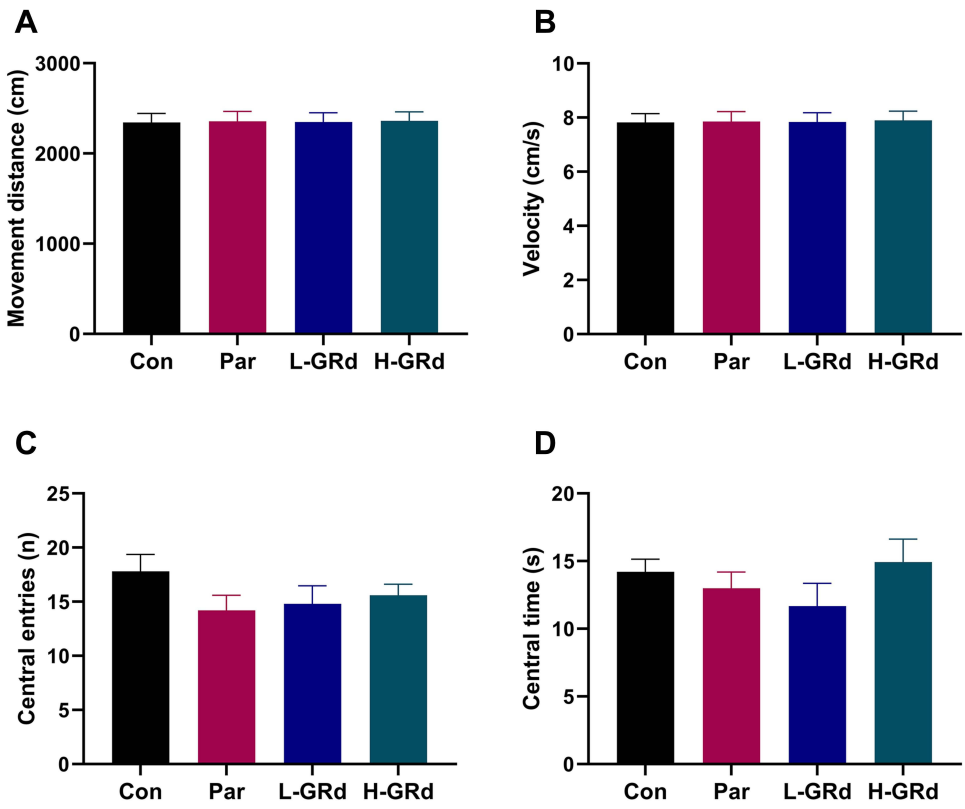

Figure 2 The effects of GRd on the OFT. (**A**) The movement distance. (**B**) The movement velocity. (**C**) The frequency of entering the center. (**D**) The duration of stay in the center. Data are expressed as mean ± SEM (n = 10/group). The results showed no difference between the different group.

Effects of GRd on OFTs

The OFT is also a classic behavioral test. The movement distance, velocity, frequency of entering the center, and the duration of stay in the center of CUMS group were all lower than the control group (Figure 7; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). However, the observation indexes of OFTs in GRd treatment group were better than in the CUMS group (Figure 7; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

SPT and OFT data showed that the CUMS model was successfully replicated and that GRd reverses CUMS-induced depression-like behavior to exhibit a significant antidepressant-like effect.

Effects of GRd on the HIF-1α-VEGF Signaling Pathway

RT-PCR and Western blotting were used to measure mRNA and protein expression of HIF-1α and VEGF, respectively. The data show that mRNA and protein expression of HIF-1α and VEGF in the CUMS group were significantly downregulated compared to the control group (Figure 8; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$). Both low- and high-dose GRd significantly increased mRNA expression of HIF-1α and VEGF, and HIF-1α protein expression in CUMS rats (Figure 8; $^{**}p < 0.01$; $^{***}p < 0.001$). In addition, high-dose GRd significantly increased VEGF protein expression in the CUMS rats (Figure 8; $^{**}p < 0.01$).

Effects of GRd on mRNA and Protein Expression of VEGFR-2

VEGFR-2, a high-affinity receptor of VEGF, is implicated in depression. We measured mRNA and protein expression of VEGFR-2 to understand whether GRd can act on VEGFR-2 to exert its antidepressant-like effects. The data showed that hippocampal mRNA and protein expression of VEGFR-2 in the CUMS group were severely downregulated compared to the control group (Figure 9; $^{\#\#}p < 0.01$). Following treatment with low-dose GRd, VEGFR-2 protein expression increased significantly (Figure 9; $^{***}p < 0.001$). Further, treatment with high-dose GRd, significantly increased both mRNA and

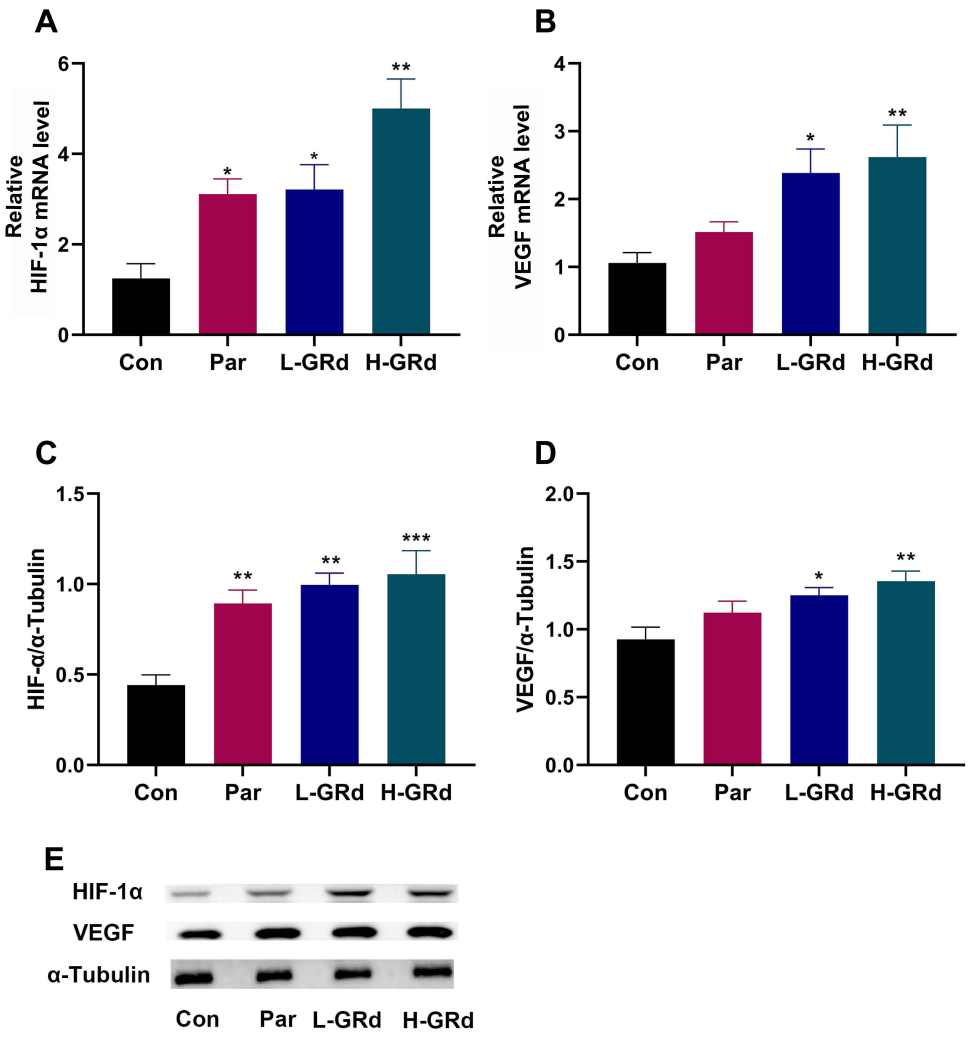

Figure 3 The effects of GRd on HIF-1α-VEGF signaling pathway. (**A**) HIF-1α mRNA expression. (**B**) VEGF mRNA expression. (**C**) HIF-1α protein expression. (**D**) VEGF protein expression. (**E**) The Western blot bands. Data are expressed as mean ± SEM (n = 6/group). **p* < 0.05 vs control group; ***p* < 0.01 vs control group; ****p* < 0.001 vs control group.

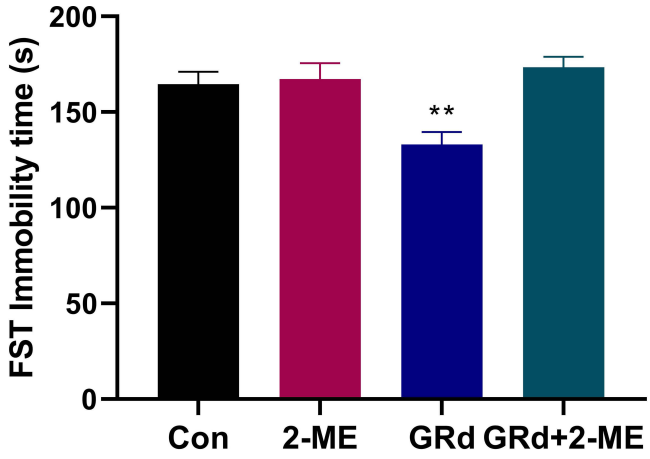

Figure 4 The effects of 2-ME on immobility time in the FST. Data are expressed as mean ± SEM (n = 10/group). ***p* < 0.01 vs control group.

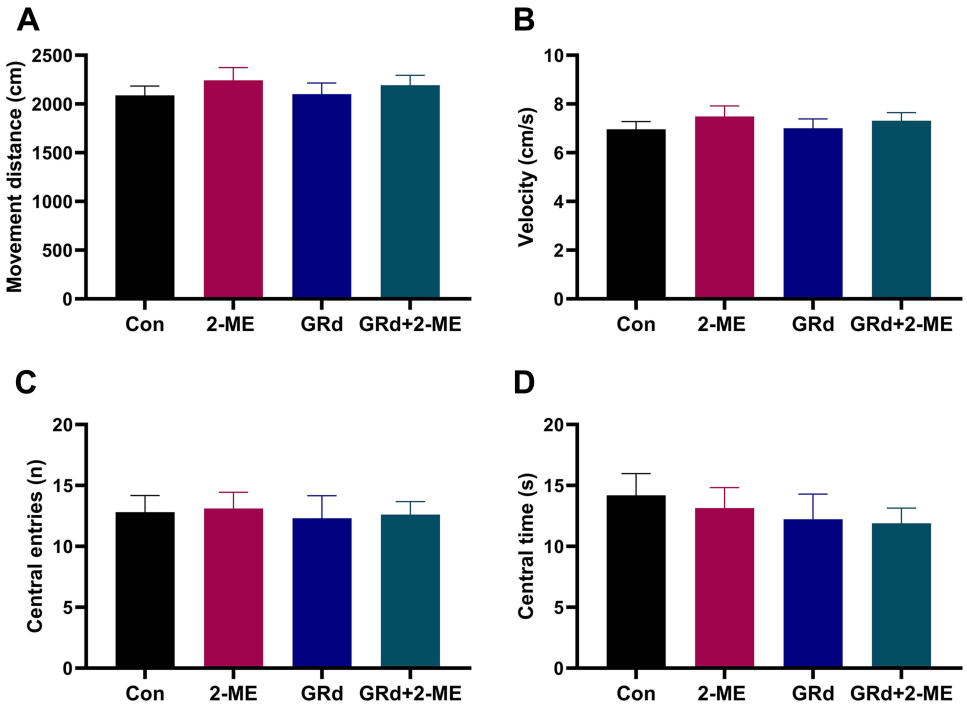

Figure 5 The effects of 2-ME on the OFT. (**A**) The movement distance. (**B**) The movement velocity. (**C**) The frequency of entering the center. (**D**) The duration of stay in the center. Data are expressed as mean ± SEM (n = 10/group). The results showed no difference between the different group.

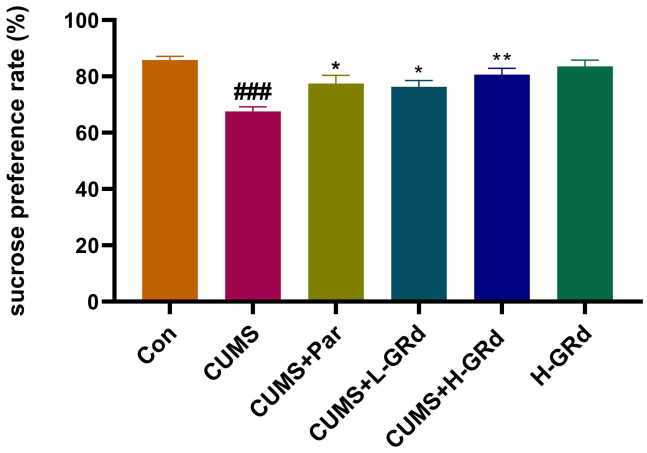

Figure 6 The effects of GRd on sucrose preference tests. Data are expressed as mean ± SEM (n = 10/group). $^{\#\#\#}p < 0.001$ vs control group. $^{*}p < 0.05$ vs CUMS group; $^{**}p < 0.01$ vs CUMS group.

protein levels of VEGFR-2 (Figure 9; $^{*}p < 0.05$; $^{***}p < 0.001$). These data confirm the involvement of VEGFR-2 in the antidepressant-like effect of GRd.

Effects of GRd on PI3K/AKT/mTOR Pathway

We used RT-PCR and Western blotting to explore VEGF- and VEGFR-2-mediated changes in mRNA and protein expression in the PI3K/AKT/mTOR signaling pathway, respectively. Our data showed that the mRNA expression of *PI3K, AKT*, and *mTOR*, and the protein expression of p-PI3K, p-AKT, and p-mTOR in the hippocampus of CUMS model rats were significantly lower compared to the control group (Figure 10; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). Moreover, low-dose GRd significantly increased mRNA expression of *AKT* and p-PI3K, p-AKT, and p-mTOR protein expression in the hippocampus of CUMS rats (Figure 10; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). High-dose GRd significantly increased

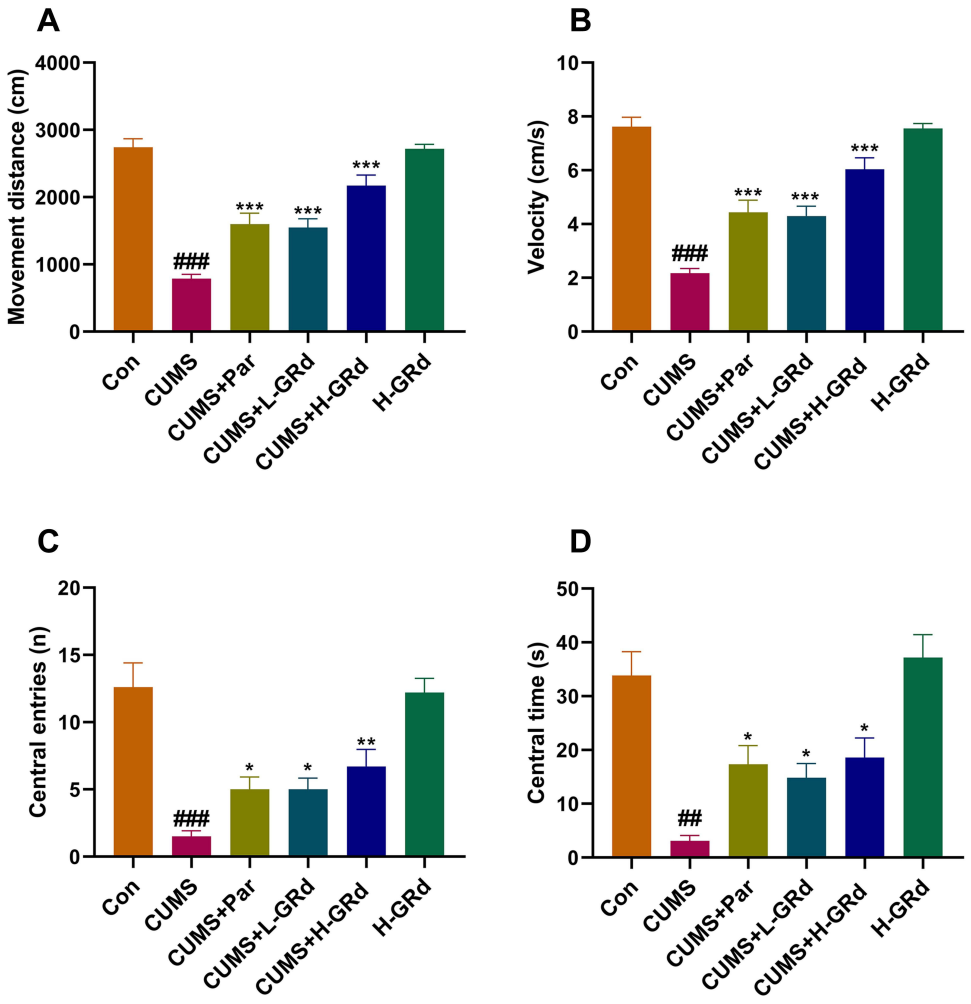

Figure 7 The effects of GRd on open field tests. (**A**) The movement distance. (**B**) The movement velocity. (**C**) The frequency of entering the center. (**D**) The duration of stay in the center. Data are expressed as mean ± SEM (n = 10/group). $^{\#\#}p < 0.01$ vs control group. $^{\#\#\#}p < 0.001$ vs control group. $^{*}p < 0.05$ vs CUMS group; $^{**}p < 0.01$ vs CUMS group. $^{***}p < 0.001$ vs CUMS group.

mRNA expression of *PI3K, AKT* and *mTOR*, while also upregulating p-PI3K, p-AKT, and p-mTOR protein expression (Figure 10; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Herein, we confirm the involvement of the PI3K/AKT/mTOR signaling pathway in GRd-induced antidepressant-like effects.

Effects of GRd on Synaptic Plasticity-Related Regulators

We also sought to understand whether GRd exerts an antidepressant-like effect by regulating synaptic plasticity-related regulators (ie SYN 1, PSD 95). We found that compared to the control group, SYN 1 and PSD 95 protein expression were significantly reduced (Figure 11; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.01$). This could be reversed by low- and high-dose GRd (Figure 11; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). These data suggest that GRd may exert its antidepressant-like effects by regulating the expression of synapse-related regulatory factors.

Molecular Docking of GRd with HIF-1α, VEGF, and VEGFR-2

A negative binding energy between a ligand and receptor is generally suggestive of spontaneous binding. The smaller the binding energy, the greater the docking activity and the greater the possibility of action. A binding energy below −5 kcal/mol is indicative of strong protein-ligand binding. Our molecular docking data suggest that the binding energy between GRd and HIF-1α, VEGF, VEGFR-2 were all less than −7 kcal/mol (−7.2, −8.3, −7.8 kcal/mol, respectively). This is indicative of a high likelihood of spontaneous binding. PyMOL was used to visualize these modelled binding data. The

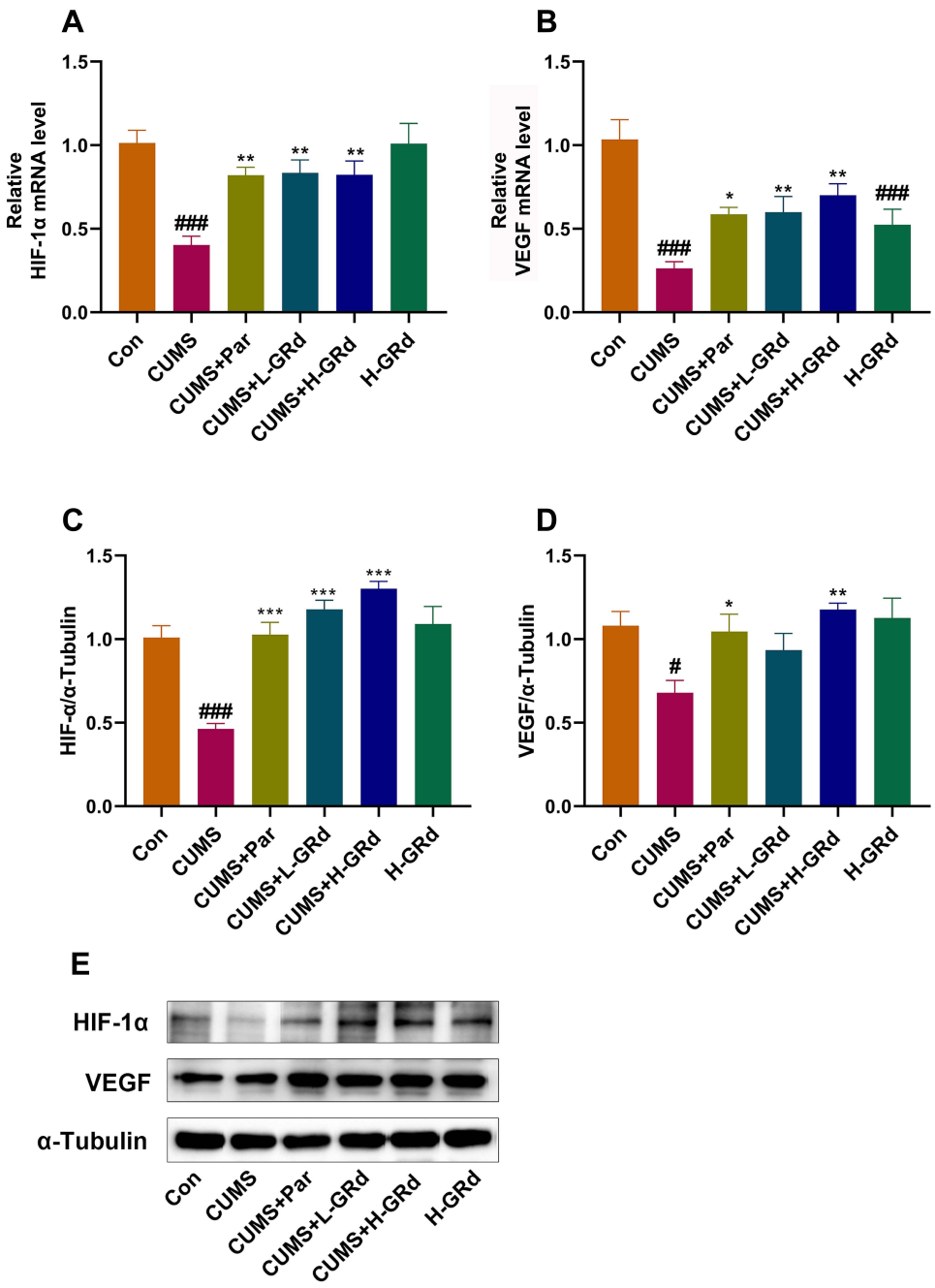

Figure 8 The effects of GRd on HIF-1α-VEGF signaling pathway. (**A**) HIF-1α mRNA expression. (**B**) VEGF mRNA expression. (**C**) HIF-1α protein expression. (**D**) VEGF protein expression. (**E**) The Western blot bands. Data are expressed as mean ± SEM (n = 6/group). $^{\#}p < 0.05$ vs control group. $^{\#\#\#}p < 0.001$ vs control group. $^{*}p < 0.05$ vs CUMS group; $^{**}p < 0.01$ vs CUMS group. $^{***}p < 0.001$ vs CUMS group.

number of hydrogen bonds formed between GRd and HIF-1α, VEGF, and VEGFR-2 are 7, 7, and 10, respectively. The amino acid residues involved in these interactions are shown in Table 2. Further, molecular docking patterns of GRd with target proteins are shown in Figure 12.

Discussion

Many ginsenosides are not easily absorbed from the intestine into the blood. Therefore, these components require metabolism by the microbiota in the gastrointestinal tract to be broken down into more easily absorbed compounds. Studies have shown that the antidepressant effect of red ginseng depends on the metabolite GRd being absorbed into the

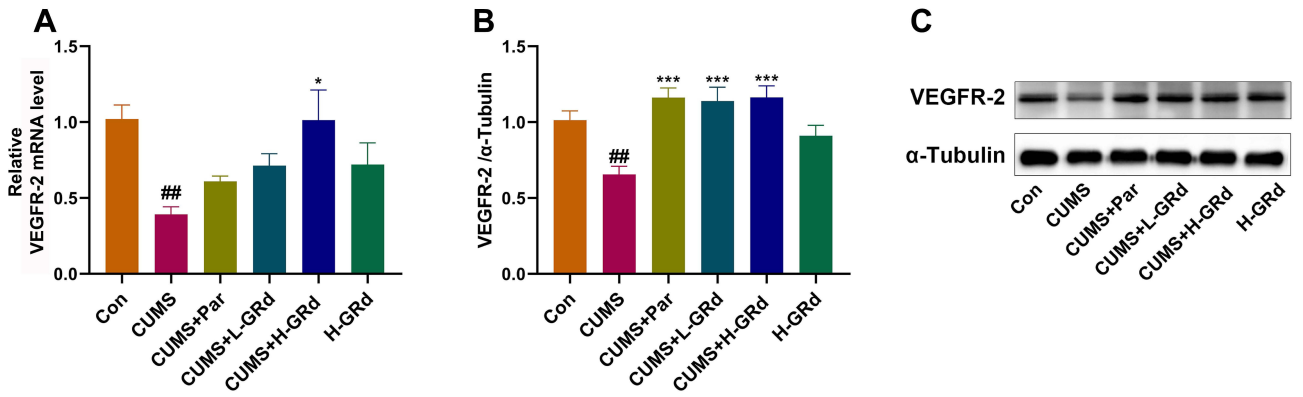

Figure 9 The effects of GRd on mRNA and protein expression of VEGFR-2. (**A**) VEGFR-2 mRNA expression. (**B**) VEGFR-2 protein expression. (**C**) The Western blot bands. Data are expressed as mean ± SEM (n = 6/group). $^{\#\#}p < 0.01$ vs control group. $^{*}p < 0.05$ vs CUMS group; $^{***}p < 0.001$ vs CUMS group.

blood. Moreover, GRd has the highest absorption rate in the blood following oral administration of red ginseng in human and mice.[22] Our previous studies have shown that Ginsenoside Rb1 (GRb1) induces antidepressant activity. GRd, a metabolite of GRb1, which is produced by removal of a β-D-glucose, was also identified in the blood, brain, feces, and urine of normal and depressed mice after administration of GRb1. In addition, our network pharmacological analysis results showed that GRd is implicated in antidepressant activity.[30]

The behavioral despair mouse model of depression is a proven depression model which places animals in irresistible adversity to breed despair. The immobility of animals after forced swimming stress is sensitive to antidepressants, making it an ideal behavioral experiment for preliminarily testing of the pharmacodynamics of antidepressants.[31,32] Compared to the control group, both high and low doses of GRd can significantly shorten the forced swimming immobility time. This suggests that GRd improves the behavioral despair of mice and exerts a significant antidepressant-like effect.

The CUMS depression model simulates unpredictable stress events and induces various long-term physical, behavioral, neurochemical, and neuroendocrine changes, similar to those observed in depressed patients.[33,34] Exposure to CUMS can reduce the movement distance, velocity, frequency of entering the center, the duration of stay in the center in OFTs, and the sucrose preference rate in SPTs, which shows that CUMS depression model is successfully reproduced. Both high and low doses of GRd reversed the depression-like behavior of CUMS rats and showed a significant antidepressant effect.

HIF-1, a base-helix-loop-helix PAS heterodimer composed of active subunit HIF-1α and constitutive subunit HIF-1β, mediates the adaptive response of cells to hypoxia. The transcriptional activity and protein stability of HIF-1α are extremely sensitive to changes in oxygen concentration. Moreover, HIF-1α is crucial for maintaining the integrity and function of HIF-1.[35] Under conditions of hypoxia, HIF-1α binds to hypoxia response elements (HREs) on a variety of target genes to induce transcription of target genes. Many studies have shown that HIF-1α, not only in response to hypoxic stress but also in response to severe psychoemotional stress, is a promising target for the treatment of depression. HIF-1α prevents oxygen homeostasis disorders, promotes neuroplasticity, and exerts neuroprotective effects by activating a series of target genes involved in regulation of angiogenesis, vasomotor control, energy and ion metabolism, erythropoiesis, and apoptosis. Triggering these protective mechanisms may have a beneficial impact on the depression, thereby preventing the development of a depressive state caused by severe psychological and emotional stress.[36–38]

Recently, many studies have shown that VEGF, a secreted angiogenic mitogen regulated by HIF-1α, plays a beneficial role in the treatment of depression.[39,40] Long-term stress decreases cell proliferation near the vascular system and expression of VEGF. Decreased expression of VEGF can be seen in the prefrontal cortex and hippocampus of patients with chronic stress-induced major depressive disorder. Further, VEGF protein expression in the cerebrospinal fluid of individuals who have attempted suicide is significantly lower compared to healthy people.[41–43] In comparison, intracerebroventricular infusion of VEGF provides an exceedingly effective antidepressant-like effect.[44] This antidepressant effect is associated with the ability of VEGF to improve cognitive function, promote neurogenesis, and facilitate

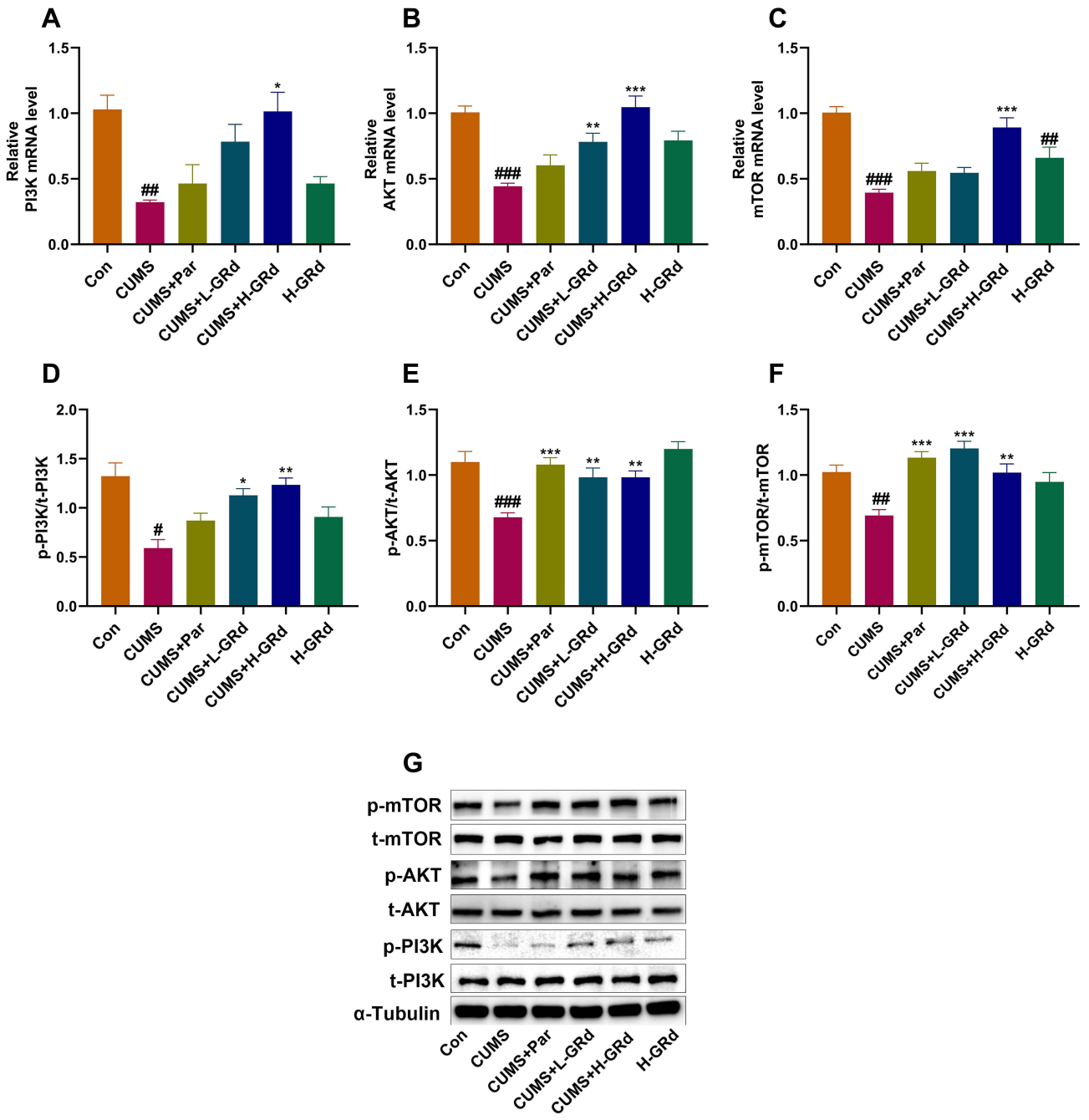

Figure 10 The effects of GRd on PI3K/AKT/mTOR signaling pathway. (**A**) PI3K mRNA expression. (**B**) AKT mRNA expression. (**C**) mTOR mRNA expression. (**D**) p-PI3K protein expression. (**E**) p-AKT protein expression. (**F**) p-mTOR protein expression. (**G**) The Western blot bands. Data are expressed as mean ± SEM (n = 6/group). $^{\#}p < 0.05$ vs control group. $^{\#\#}p < 0.01$ vs control group. $^{\#\#\#}p < 0.001$ vs control group. $^{*}p < 0.05$ vs CUMS group; $^{**}p < 0.01$ vs CUMS group. $^{***}p < 0.001$ vs CUMS group.

hippocampal synaptic plasticity.[45–48] Comprehensively, activation of the HIF-1α-VEGF signaling pathway may be implicated in the treatment of depression, which is highly consistent with our experimental results. Compared to the CUMS group, high and low doses of GRd can significantly increase mRNA and protein expression of hippocampal HIF-1α and VEGF in both the behavioral despair mouse model of depression and the rat CUMS model.

Intriguingly, 2-ME, a natural metabolite of estradiol and an effective HIF-1α inhibitor, inhibits the transcriptional activity and protein expression of HIF-1α by inducing microtubule depolymerization and the production of reactive oxygen species.[49,50] In our HIF-1α inhibition experiment, the forced swimming immobility time of mice treated with

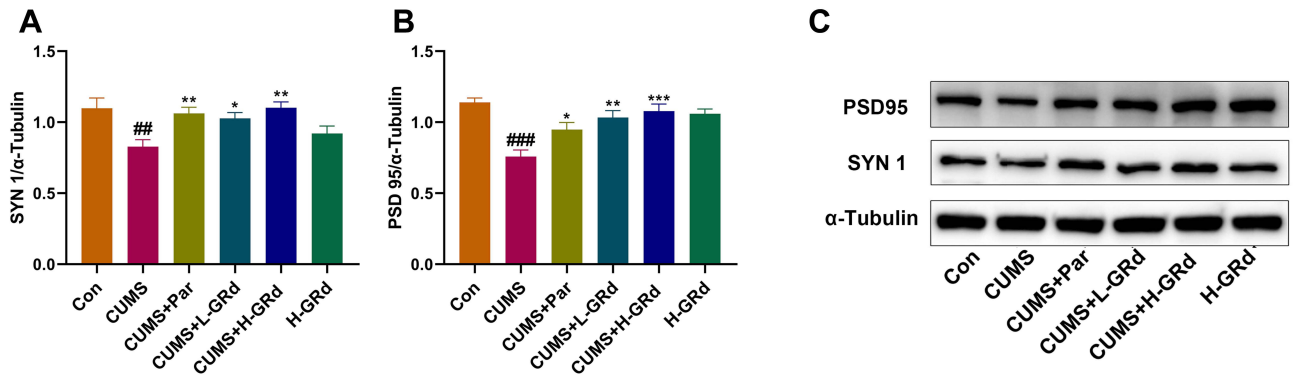

Figure 11 Effects of GRd on synaptic plasticity-related regulators. (**A**) SYN 1 protein expression. (**B**) PSD 95 protein expression. (**C**) The Western blot bands. Data are expressed as mean ± SEM (n = 6/group). $^{\#\#}p < 0.01$ vs control group. $^{\#\#\#}p < 0.001$ vs control group. $^{*}p < 0.05$ vs CUMS group; $^{**}p < 0.01$ vs CUMS group. $^{***}p < 0.001$ vs CUMS group.

2-ME following oral administration of GRd was not shorter than the control group. This indicates that the antidepressant effect of GRd can be blocked by 2-ME, further confirming that HIF-1α is essential for the antidepressant effect of GRd.

The antidepressant effect of GRd benefits from the increased expression of hippocampal the synaptic plasticity-related regulators, SYN 1 and PSD 95. There is increasing evidence to suggest that synaptic plasticity disorder may be a pathological mechanism involved depression. Moreover, an improvement in synaptic plasticity plays a critical role in the treatment and prognosis of depression.[51–53] Synaptic plasticity is generally coordinated with the synapse-associated proteins, mainly including presynaptic SYN 1 and postsynaptic PSD 95. SYN 1, a neuron-specific phosphoprotein associated with synapses, is distributed in almost all nerve terminals and located on the surface of the presynaptic vesicle membrane. SYN 1 regulates the transportation and circulation of synaptic vesicles to promote neurodevelopment, neuronal information transmission, and synaptic plasticity.[54–56] PSD 95, a protein located on the cytoplasmic side of the postsynaptic membrane and related to synaptic signal transmission, plays a significant role in regulating neuron survival and synaptic function. Further, PSD 95 is widely involved in the onset and treatment of cognitive and emotional related mental diseases.[57,58] Many studies have shown that protein expression of SYN 1 and PSD 95 are decreased in the prefrontal cortex of patients with major depression. A decrease of presynaptic SYN 1 and postsynaptic PSD 95 in the hippocampus and prefrontal cortex due to chronic stress may lead to depressive behavior and a severe decline in cognitive function in rodents.[59,60] Conversely, increased SYN 1 and PSD 95 expression can improve depression.[61,62] Consistent with our findings, GRd greatly reversed the significant decrease in SYN 1 and PSD 95 protein levels in the hippocampus of CUMS rats. This shows that the antidepressant-like effect of GRd is linked to the expression of hippocampal synaptic plasticity-related regulators.

There is a close correlation between the activation of the HIF-1α-VEGF signaling pathway and increased expression of synaptic plasticity-related regulatory factors. Both are beneficial for improving depression.[63–65] Our results show that the activation of the HIF-1α-VEGF signaling pathway may promote the expression of synaptic plasticity-related regulatory factors through regulation of the PI3K-AKT-mTOR signaling transduction pathway mediated by vascular

Table 2 Binding Affinity Between GRd and Protein Targets

Ligand	Docking Target	H Bonds	Amino Acid	Binding Energies (kcal \Mol)
GRd	HIF-1α (PDB: 1L8C)	7	ASP-93; ASP-100; GLN-147; LEU-101; LYS-75; TRP-74	−7.2
	VEGF (PDB: 3QTK)	7	ARG-49; GLV-57; GLY-58; THR-64	−8.3
	VEGFR-2 (PDB: 3VHE)	10	ALA-1050; ARG-1051; ASN-923; ASP-1056; ASP-1058; GLU-872; LYS-871; SER-925	−7.8

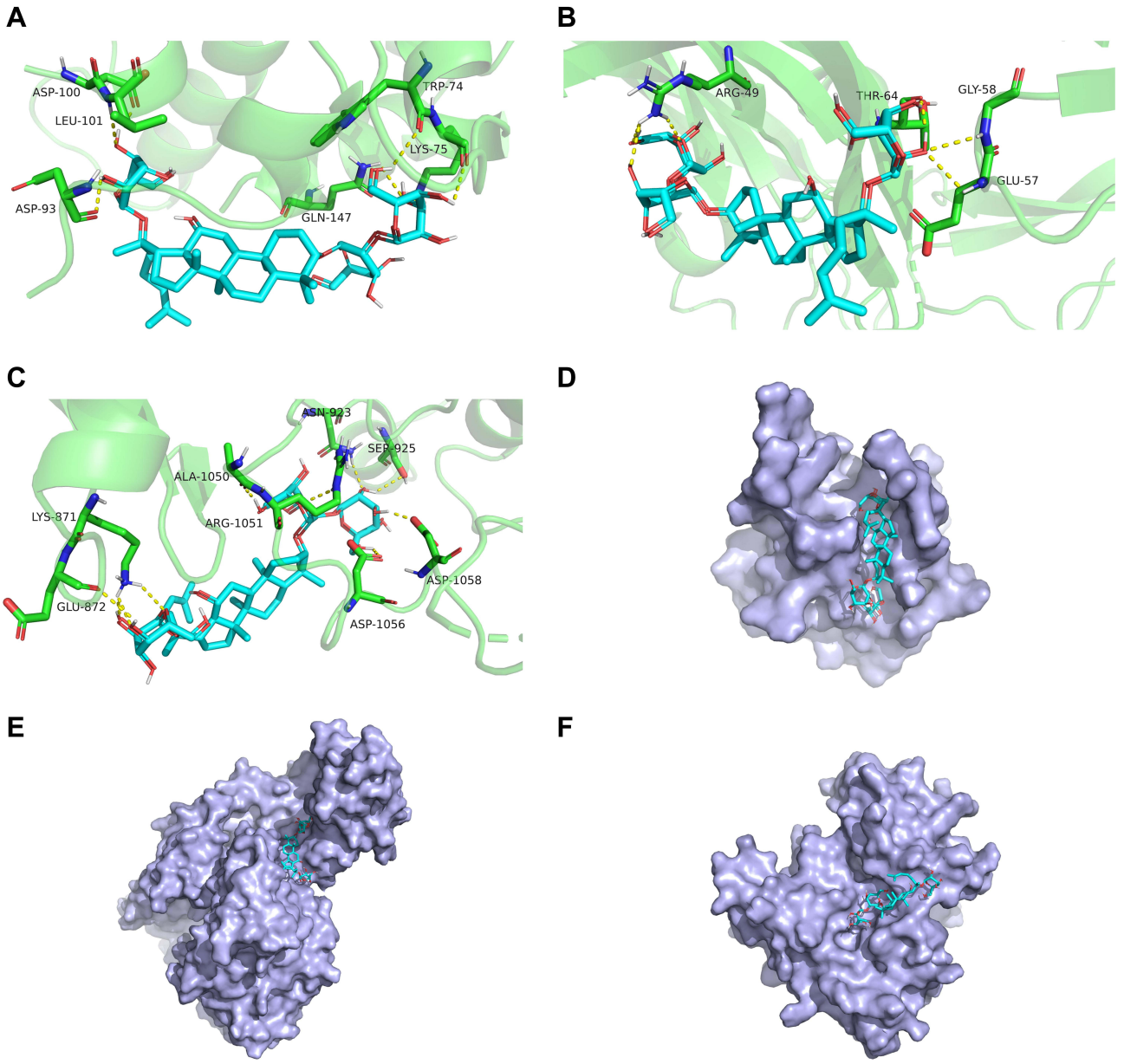

Figure 12 Molecular docking patterns of GRd with target proteins. Molecular docking patterns of GRd with target proteins such as HIF-1α (**A** and **D**); VEGF (**B** and **E**); VEGFR-2 (**C** and **F**).

endothelial growth factor receptor 2 (VEGFR-2, also known as Flk-1). The downstream signal transduction molecular mechanism of VEGF is complex. VEGFR-2, a high-affinity receptor tyrosine kinase of VEGF, which is highly expressed in the rodent hippocampus, can activate the PI3K signaling pathway to stimulate cell proliferation and survival.[66,67] VEGF-Flk-1 signaling brings into play a considerable role for synaptic plasticity in hippocampal dependent processes, such as learning and memory, and regulating synaptic transmission. These effects are important in the behavioral responses of animal models receiving multiple antidepressants and can, therefore, be used as valuable predictors of antidepressant activity.[68–71]

Mammalian target of rapamycin (mTOR), one of the downstream signaling molecules of PI3K, is activated and regulated by the PI3K-AKT pathway, which is a pivotal target for glutamatergic and cholinergic drugs which exert rapid antidepressant effects.[72–74] Studies have shown that the mTOR signal is closely related to synaptic plasticity. When the signal is stimulated, it will rapidly increase the expression of synaptic proteins (eg PSD 95 and SYN 1) and the number

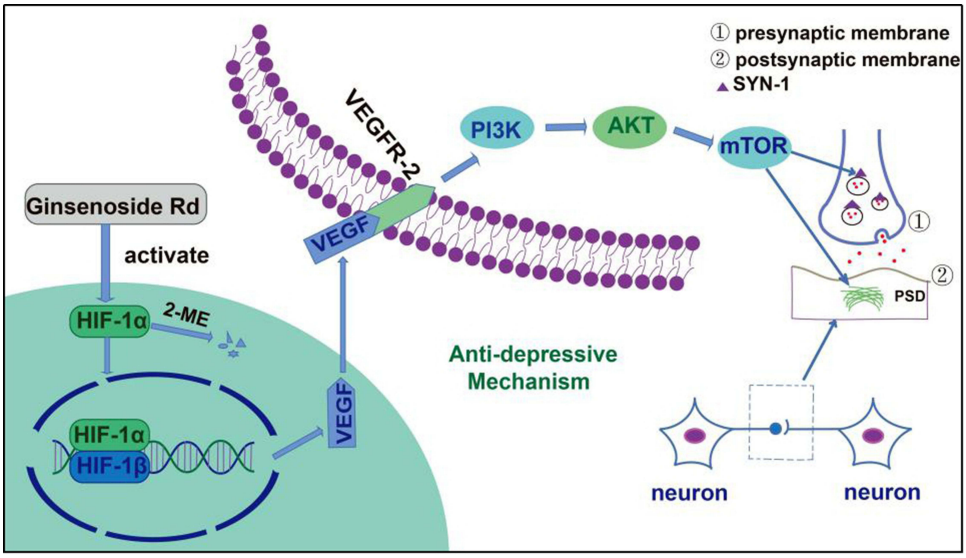

Figure 13 The antidepressant mechanism of GRd.

of spinal synapses,[75–77] which is consistent with our research results. After CUMS rats were subjected to chronic stress, we found that mRNA and protein expression of VEGFR-2, PI3K, AKT, and mTOR were significantly reduced. These can all be reversed by GRd.

In recent years, molecular docking has become an important technology in the field of computer-aided drug design. This method is a theoretical simulation method for studying the interactions between molecules (eg ligands and receptors) and predicting their binding mode(s) and affinity.[78] Our molecular docking studies revealed additional insights into the effects of GRd on HIF-1α signaling pathway related target proteins for therapeutic intervention in depression. In the present study, a molecular docking study of GRd (ligand) was performed against focused target proteins including HIF-1α, VEGF, and VEGFR-2. Our data reveal that GRd docks well into HIF-1α, VEGF, and VEGFR-2 with docking scores of −7.2, −7.8, and −8.3 kcal/mol, respectively. This suggests that all three proteins may have a high binding affinity for GRd. We also analyzed the hydrogen bonds between GRd and the target protein. The number of hydrogen bonds formed by GRd with HIF-1α, VEGF, and VEGFR-2 were 7, 7 and 10, respectively. The details of the binding amino acid residues are shown in Table 2. In addition, the molecular docking patterns of GRd with the target protein are shown in Figure 12. The modelled antidepressant mechanism of GRd is shown in Figure 13.

Conclusion

Our data indicate that GRd exhibits a significant antidepressant-like effect. The observed beneficial effects may be attributed to the activation of the HIF-1α-VEGF signaling pathway, VEGFR-2-mediated PI3K-AKT-mTOR signaling pathway, and increased expression of the synaptic plasticity-related proteins, SYN 1 and PSD 95.

Abbreviations

2-ME, 2-methoxyestradiol; CUMS, chronic unpredictable mild stress; EPO, erythropoietin; FST, forced swimming tests; GRd, ginsenoside Rd; HIF-1α, hypoxia-inducible factor-1α; HREs, hypoxia response elements; mTOR, mammalian target of rapamycin; OFT, open field tests; PSD 95, postsynaptic density protein 95; SPT, sucrose preference tests; SSRIs, selective serotonin reuptake inhibitors; SYN 1, synapsin 1; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2.

Institutional Review Board Statement

The study was approved by The Experimental Animal Ethics Committee of the Academic Committee of Beijing University of Chinese Medicine (project identification code: BUCM-2021082802-3002).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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