Piperlongumine produces antidepressant-like effects in rats exposed to chronic unpredictable stress

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Piperlongumine, an alkaloid compound extracted from *Peper longum* L, has been reported to produce neuroprotective effects in the brain and exert various pharmacological activities such as antitumor, antiangiogenic, anti-inflammatory and analgesic properties. The aim of this study was to investigate the antidepressant-like effects and the possible mechanism of action of piperlongumine in a chronic unpredictable stress (CUS) model. We found that, with venlafaxine as a positive control, orally administered piperlongumine (12.5 and 25 mg/kg) for 7 days, not a single dose, significantly reduced immobility time in the forced swimming test, but did not alter locomotor activity in the open field test, indicating that piperlongumine has antidepressant-like effects without nonspecific motor changes. Then, using the CUS model of depression, piperlongumine was administrated orally for 4 weeks, followed by sucrose preference and forced swimming tests to evaluate the depressive-like behaviors. We found that piperlongumine reversed both the decreased sucrose preference and increased immobility time in rats exposed to CUS. In addition, piperlongumine also reversed the increase in proinflammatory cytokine levels in the hippocampus of rats in the CUS model. Altogether, the present study demonstrated that piperlongumine exhibits the antidepressant-like effects in rats, which may be mediated by the inhibition of the neuronal inflammation in the hippocampus. *Behavioural Pharmacology* 30:721–728
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Introduction

Depression is a common and debilitating illness that is becoming a leading cause of disability and disease burden worldwide. Currently, available antidepressant medications have mostly targeted the monoamine neurotransmitter systems and are associated with high rates of partial responsiveness or non-responsiveness (Duman *et al.*, 2016; Ionescu and Papakostas, 2017). Esketamine was recently reported to exert rapid antidepressant effect and was the first glutamatergic drug approved by the US Food and Drug Administration for treating patients with treatment-resistant depression (Daly *et al.*, 2018; Molero *et al.*, 2018). But this drug is only available through a restricted distribution system because of the risk of serious adverse effects and abuse potential. The development of novel efficient antidepressants has been hampered as the underlying etiology and pathophysiology of depression still remains largely unclear.

Growing evidence indicates that immune and endocrine interactions may contribute to the emergence and development of depression (Kim and Won, 2017; Lima-Ojeda *et al.*, 2018). The immune system affects the central nervous system through cytokines, which induce alterations in brain structure and function in major depressive disorder (Jeon and Kim, 2018; Kim and Won, 2017). Growing evidence indicates that proinflammatory cytokines, including interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, increased and anti-inflammatory cytokines, including IL-4, IL-10 and TGF-β, decreased in depressed patients (Sutcigil *et al.*, 2007; Dowlati *et al.*, 2010; Jeon and Kim, 2016). Preclinical studies also showed that chronic stress increased the production of proinflammatory cytokines and reactive oxygen species, thus inducing neuronal atrophy and dysfunction by increasing neurotoxic metabolites, or directly exerting neurotoxic effects on specific brain regions (D'Mello and Swain, 2017; Kim and Won, 2017; Liberman *et al.*, 2018). The levels of certain cytokines also mediate the behavioral actions of antidepressants (Alboni *et al.*, 2013; Réus *et al.*, 2017).

Piperlongumine is an alkaloid isolated from the long pepper *Piper longum* L, which has been studied in the context of Parkinson's disease (Wang *et al.*, 2016; Liu *et al.*, 2018), experimental autoimmune encephalomyelitis (Gu *et al.*, 2017), rheumatoid arthritis (Xiao *et al.*, 2016) and stroke (Yang *et al.*, 2014), and the preliminary results suggest a possible relevance under these pathological conditions. Piperlongumine also exhibits a variety of pharmacological effects and pharmacological activities, such as antitumor, antiplatelet, antinociceptive and anti-inflammatory

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properties (Bezerra *et al.*, 2013; Piska *et al.*, 2018; Song *et al.*, 2018). Previous studies showed that piperlongumine inhibited lipopolysaccharide-induced neuroinflammation, and reduced the production of proinflammatory cytokines (e.g., TNF-α and IL-6) and reactive oxygen species by suppressing the nuclear factor kappa B signaling pathway (Rodrigues Silva *et al.*, 2008; Lee *et al.*, 2013; Gu *et al.*, 2018; Kim *et al.*, 2018). Oral administration of piperlongumine for 8 weeks improved cognitive function and increased the neurogenesis in the dentate gyrus of the hippocampus in aged mice (Go *et al.*, 2018). A previous study also showed that piplartine presents antidepressant effect in the forced swimming test (FST) and exerts anxiolytic effect in the elevated plus maze test in mice (Cícero Bezerra Felipe *et al.*, 2007).

In the present study, we investigated whether oral piperlongumine administration produces antidepressant-like effects in the FST and in the rat depression model of chronic unpredictable stress (CUS). We also assessed hippocampal inflammation by measuring TNF-α, IL-1β and IL-6 levels to clarify the potential mechanism of action of piperlongumine.

Methods

Subjects

Male Sprague–Dawley rats (240–260 g) were obtained from the Laboratory Animal Center, University of South China. All experimental procedures were approved by the University of South China and carried out in accordance with the corresponding guidelines. The rats were housed in groups of four or five per cage under a normal 12 hours/12 hours light to dark schedule with lights on at 07:00 a.m. Ambient temperature and relative humidity were maintained at 23 ± 2 °C and at 50 ± 5%, respectively. Food and water were freely provided throughout the experiments. A total of 166 rats were used in the experiments, and all behavioral tests were conducted during the dark hours of rats. Our estimates of the number of animals needed for the behavioral tests ($n \geq 8$ per group), and enzyme-linked immunosorbent assays ($n \geq 6$ per group) were based on previous studies (Zhu *et al.*, 2012; Dong *et al.*, 2018).

Open field test

Locomotor activity was measured by the open field test (OFT). The apparatus consisted of a 75 cm × 75 cm square × 40 cm height arena that was divided into 25 equal squares on the floor. An individual rat was placed in the center of the cage, and the number of crossings was counted for 5 minutes by researchers who were blind to the experimental conditions.

Forced swimming test

The rats were placed in a plastic cylinder (25 cm diameter × 50 cm height) that was filled with 23–25°C water to a depth of 45 cm. On the first day, each rat was placed in the cylinder for 15 minutes for habituation and then removed, dried, and returned to its home cage. Twenty-four hours later, the test was videotaped for 5 minutes, and immobility time was measured. The definition of immobility was the absence of all movements with the exception of motions required to maintain the animal's head above the water. Observers were blind to the treatment group of the rats.

Chronic unpredictable stress

The procedure of CUS was performed as previously described (Xu *et al.*, 2017). The rats were housed in groups of four or five per cage, and were exposed to a variable sequence of unpredictable stressors for 28 consecutive days (two stressors per day). In detail, the stressors included water deprivation, soiled cage exposure, light/dark succession (2 hours), empty bottle exposure, 45° cage tilt, space reduction, predator sounds, overnight illumination, tail clamp (1 minute), forced cold swim (4ºC, 5 minute), vibration (1 hour) and restraint (1 hour). The control rats were housed in a separate room and received handling for 5 minutes each day. To ensure the unpredictability of the stressors, two stressors were randomly applied per day. During the CUS procedure, rats that were severely sick or died were excluded from the experiment ($n = 4$).

Sucrose preference test

The rats were individually housed and trained to be adapted to sucrose solution (1%, w/v) in two bottles for 48 hours. After adaptation, the rats were deprived of water for 6 hours. During the test, the rats had free access to two bottles that contained 1% sucrose or tap water, respectively, for 1 hour. The volumes of the consumed sucrose solution and water were recorded. Sucrose preference was defined as the following: sucrose consumption/(sucrose consumption + water consumption) × 100%.

Enzyme-linked immunosorbent assay

Rats were sacrificed by decapitation 24 hours after the last stress. Whole brains were rapidly removed and were immediately frozen in liquid nitrogen and then stored at –80°C until assay. The hippocampal tissue was obtained from ~1-mm-thick coronal sections and homogenized in PBS (0.01 M, pH 7.4) with protease and phosphatase inhibitor (Beyotime Biotechnology, Beijing, China). Total protein extracts from the tissue were used for cytokine measurement. The proinflammatroy cytokines including IL-1β, IL-6 and TNF-α levels in the hippocampus were measured by commercial ELISA kits according to the manufacturer's instructions.

Experimental design

Experiment 1: effects of acute piperlongumine treatment on the locomotor activity in open field test and the immobility in forced swimming test in rats

The rats were habituated for 5 days, and were randomly divided into five groups: vehicle ($n = 9$), 6.25 mg/kg

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piperlongumine ($n = 11$), 12.5 mg/kg piperlongumine ($n = 9$), 25 mg/kg piperlongumine ($n = 10$) and 10 mg/kg venlafaxine ($n = 10$). A single dose of the drugs was given orally 55 minutes before the OFT and 1 hour before the FST.

Experiment 2: effects of piperlongumine treatment for 7 days on the locomotor activity in open field test and the immobility in forced swimming test in rats

A separate group of rats were habituated for 5 days, and were randomly divided into five groups: vehicle ($n = 10$), 6.25 mg/kg piperlongumine ($n = 11$), 12.5 mg/kg piperlongumine ($n = 9$), 25 mg/kg piperlongumine ($n = 10$) and 10 mg/kg venlafaxine ($n = 9$). The rats received the drugs orally once daily for 7 consecutive days. On day 7, 55 minutes after the last treatment, crossings in the OFT were measured for 5 minutes. Immediately after the OFT, the rats were subjected to the FST for 5 minutes.

Experiment 3: effects of piperlongumine treatment for 4 weeks on depressive-like behaviors in rats exposed to chronic unpredictable stress

A separate group of rats were randomly divided into four groups: the control-vehicle group ($n = 10$), the CUS-vehicle group ($n = 9$), the Control-piperlongumine group ($n = 8$) and the CUS-piperlongumine group ($n = 10$). After habituation for 5 days, the rats were subjected to 28 days of CUS or housed in a separate room and received handling for 5 minutes each day. At the same time, the rats received vehicle or 25 mg/kg piperlongumine orally once daily for 28 consecutive days at 12:00 a.m. Then, the rats underwent the SPT and FST to evaluate depressive-like behaviors.

Experiment 4: effects of chronic unpredictable stress and piperlongumine treatment on proinflammatory cytokine levels in the hippocampus

A separate group of rats were randomly divided into four groups: the control-vehicle group ($n = 7$), the CUS-vehicle group ($n = 7$), the control-piperlongumine group ($n = 6$) and the CUS-piperlongumine group ($n = 7$). After habituation for 5 days, the rats were subjected to 28 days of CUS or housed in a separate room and received handling for 5 minutes each day. At the same time, the rats received vehicle or 25 mg/kg piperlongumine orally once daily for 28 consecutive days at 12:00 a.m. One day after the last stress, the rats were decapitated and the brains were collected for assessment of TNF-α, IL-1β and IL-6 levels.

Drugs and reagents

Piperlongumine (purity > 98%, confirmed by high-performance liquid chromatography analysis) was purchased from BioChemPartner (Shanghai, China). Piperlongumine was dissolved in 0.5% carboxymethyl cellulose and administered by oral gavage once daily. Vehicle was 0.5% carboxymethyl cellulose and was administered by oral gavage once daily. The doses of piperlongumine were based on previous studies with minor modification (Wang *et al.*, 2016; Go *et al.*, 2018). Venlafaxine (purity > 99%) was purchased from Chengdu Daxi'nan Pharmaceutical Co., Ltd. (Chengdu, Sichuan Province, China). Venlafaxine was freshly dissolved in saline and was administered by oral gavage once daily. Commercial ELISA kits for IL-1β, IL-6 and TNF-α measurement were purchased from Boster (Wuhan, China).

Statistical analysis

All of the statistical analyses were performed using SPSS 20.0 software (SPSS, Chicago, Illinois, USA). The data are expressed as mean ± SEM. The data were analyzed using one- or two-way analysis of variance (ANOVA), followed by Tukey's post-hoc tests. A value of $P < 0.05$ was considered to be statistically significant for analysis.

Results

Piperlongumine produced antidepressant-like effect in the forced swimming test

We first investigated the antidepressant effect of piperlongumine in the FST. One-way ANOVA of the FST data revealed significant main effects of drug treatment (7 days treatment: $F_{4,44} = 8.25$, $P = 0.001$, Fig. 1a; 1 day treatment: $F_{4,44} = 2.98$, $P < 0.05$; Fig. 1b). Oral treatment with piperlongumine for 7 days at doses of 12.5 mg/kg ($P < 0.05$) and 25 mg/kg ($P = 0.001$) significantly reduced immobility time, but the 6.25 mg/kg dose had no significant effect on the immobility time compared with vehicle-treated rats. The positive control venlafaxine (10 mg/kg) significantly reduced immobility time in the FST ($P < 0.001$; Fig. 1a) compared with vehicle-treated rats. Acute treatment with a single dose of piperlongumine (6.25, 12.5 and 25 mg/kg) had no significant effect on the immobility time in the FST (Fig. 1b). A single dose of venlafaxine decreased immobility time in the FST ($P = 0.077$; Fig. 1b) compared with vehicle-treated rats.

To exclude the possibility that piperlongumine induces nonspecific locomotor alterations, we measured the effects of piperlongumine on locomotor activity in the OFT. Rats treated with piperlongumine (6.25, 12.5 and 25 mg/kg) and the positive control venlafaxine (10 mg/kg), for either 7 or 1 day, did not alter the number of crossings in the OFT (Fig. 2a and b) compared with the vehicle-treated rats, indicating that the antidepressant-like effects of piperlongumine are not attributable to a stimulatory effect on locomotor function.

Chronic administration of piperlongumine produced an antidepressant-like effect in the chronic unpredictable stress model

Then, we tested the behavioral actions of piperlongumine in the CUS model. Two-way ANOVA of the SPT data revealed a significant piperlongumine treatment × stress

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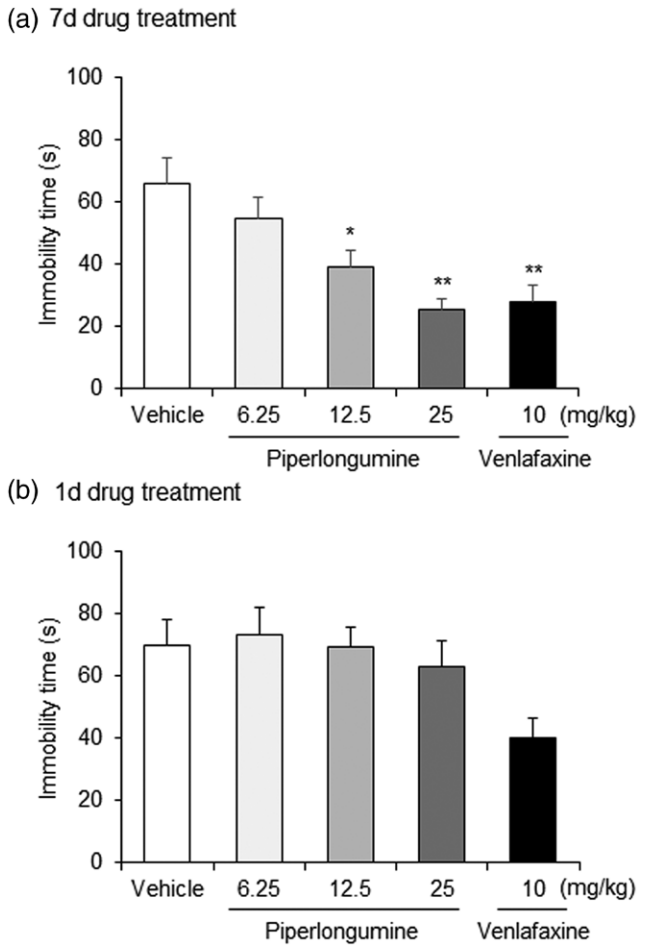
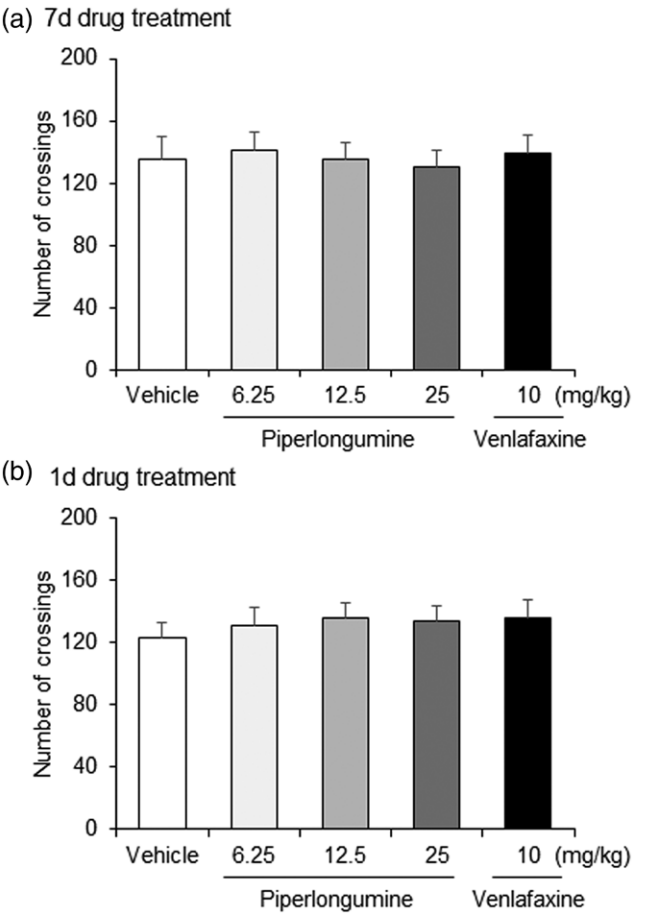
Fig. 1



Antidepressant effect of piperlongumine in the FST in rats. Oral administration of piperlongumine (12.5 and 25 mg/kg) and venlafaxine (10 mg/kg) for 7 days reduced the immobility time (a), while a single dose of piperlongumine did not alter the immobility time (b) in the FST. The rats were exposed to the FST 60 minutes after the last treatment,. Data are expressed as mean ± SEM. *P<0.05, **P<0.01, compared with the vehicle group. FST, forced swimming test.

Fig. 2



The effect of piperlongumine on locomotor activity in the OFT. Both 7 days (a) and 1 day (b) piperlongumine treatment had no effects on the number of crossings in the OFT. Piperlongumine and venlafaxine were administered orally for 7 days or a single dose, respectively. The rats were exposed to the OST 55 minutes after the last treatment,. Data are expressed as mean ± SEM. OFT, open field test.

interaction ($F_{1,33}$ = 4.54, P < 0.05; Fig. 3a) with significant main effects of both treatment ($F_{1,33}$ = 9.52, P < 0.005; Fig. 3a) and stress ($F_{1,33}$ = 16.35, P < 0.001; Fig. 3a). The post-hoc tests showed that CUS significantly decreased sucrose preference in the SPT (P < 0.001), which was reversed by oral treatment for 4 weeks with piperlongumine (25 mg/kg) (P < 0.001; Fig. 3a). Additionally, piperlongumine did not significantly alter the sucrose preference in the control group.

Two-way ANOVA of the FST data revealed significant main effects of both piperlongumine treatment ($F_{1,33}$ = 19.40, P < 0.001; Fig. 3b) and stress ($F_{1,33}$ = 12.27, P = 0.001; Fig. 3b). The post-hoc tests showed that CUS significantly increased immobility time in the FST (P < 0.005), which was reversed by 4-week treatment with piperlongumine (P < 0.001; Fig. 3b). Additionally, piperlongumine significantly decreased the immobility time in the control group (P < 0.02; Fig. 3b).

These results indicate that chronic administration of piperlongumine prevented the CUS-induced depressive-like behaviors in rats.

Chronic administration of piperlongumine decreased the proinflammatory cytokine levels in the hippocampus of rats exposed to chronic unpredictable stress

We measured the levels of proinflammatory cytokines including TNF-α, IL-1β and IL-6 in the hippocampus after CUS and piperlongumine treatment. As shown in Fig. 4, two-way ANOVA of the ELISA data revealed significant piperlongumine treatment × stress interactions for TNF-α ($F_{1,23}$ = 6.10, P < 0.025; Fig. 4a), IL-1β ($F_{1,23}$ = 7.20, P < 0.02; Fig. 4b) and IL-6 ($F_{1,23}$ = 11.98,

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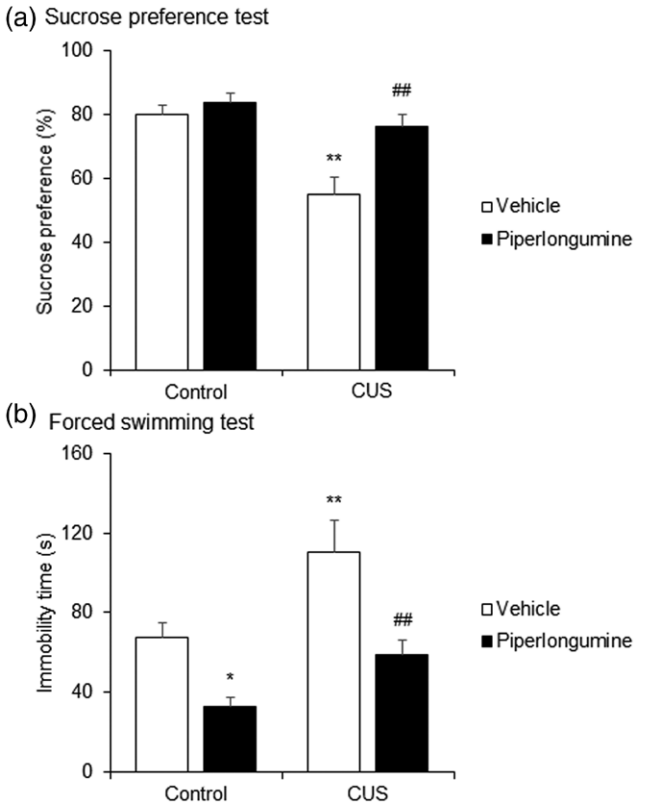
Fig. 3



Piperlongumine prevented CUS-induced depressive-like behaviors. Oral administration of piperlongumine (25 mg/kg) for 4 weeks increased sucrose preference in the SPT (a) and decreased immobility time in the FST (b) in rats exposed to CUS. $^{*}P<0.05$ and $^{**}P<0.01$, compared with the control-vehicle group. $^{\#\#}P<0.01$, compared with the CUS-vehicle group. CUS, chronic unpredictable stress; FST, forced swimming test; SPT, sucrose preference test.

$P<0.002$; Fig. 4c) levels. The post-hoc tests showed that that CUS significantly increased TNF-α ($P<0.001$), IL-1β ($P<0.001$) and IL-6 ($P<0.000$) levels in the hippocampus, which were reversed by 4-week treatment with piperlongumine. In addition, piperlongumine did not significantly alter cytokine levels in the control group.

Discussion

Piperlongumine (also known as piplartine) is a natural alkaloid compound isolated from the long pepper and is well known for its antiplatelet aggregation, anti-inflammatory, and antitumor properties (Bang *et al.*, 2009; Zheng *et al.*, 2016; Kim *et al.*, 2018; Piska *et al.*, 2018). The metabolic profile and safety of piperlongumine have been documented (Marques *et al.*, 2014; de Lima Moreira *et al.*, 2016), and orally administration of piperlongumine has been shown to be rapidly distributed throughout the brain, as measured by liquid chromatography-tandem mass spectrometry (Liu *et al.*, 2018). Several studies have revealed that piperlongumine has therapeutic potential under a variety of pathological conditions including arthritis, lupus nephritis, autoimmune encephalomyelitis, neurodegenerative diseases, and stroke (Yang *et al.*, 2014; Yao *et al.*, 2014; Prasad and Tyagi, 2016; Xiao *et al.*, 2016; Gu *et al.*, 2017; Liu *et al.*, 2018). A previous study also showed that piplartine has anxiolytic and antidepressant effects in mice (Cícero Bezerra Felipe *et al.*, 2007). In the present study, we found that chronic piperlongumine administration did not change locomotor activity in the OFT and produced antidepressant-like effects in the FST. Additionally, piperlongumine treatment for 4 weeks reversed the decrease in sucrose preference and the increase in immobility time induced by CUS, indicating that piperlongumine prevented depressive-like behaviors in the CUS model. Piperlongumine could be a promising component for the prevention and treatment of depression.

Meta-analyses of representative national samples indicate that women are twice more likely to suffer from depression than men (Salk *et al.*, 2017). Accumulating evidence has also shown different influences of sex on depressive-like behaviors and antidepressant response in multiple depression animal models (LeGates *et al.*, 2019; Ma *et al.*, 2019). However, only male rats were used in our present study. The effects of piperlongumine treatment on behaviors in females need further investigation.

Inflammation has been well recognized as a major contributor to the development of depression. Several proinflammatory cytokines are increased under conditions of stress, and they affect neurotransmitter systems, brain function and mood (Kim and Won, 2017; Finnell and Wood, 2018; Jeon and Kim, 2018). Inflammatory cytokines are closely associated with pathogenesis and treatment of depression (Kappelmann *et al.*, 2018; Himmerich *et al.*, 2019). Previous studies showed that piperlongumine inhibited adhesion and migration of leukocytes, reduced the production of proinflammatory cytokines such as TNF-α and IL-6, and exerted neuroprotective effects in the brain (Rodrigues Silva *et al.*, 2008; Lee *et al.*, 2013; Kim *et al.*, 2018). Chronic piperlongumine treatment rescued age-related cognitive impairment and improved hippocampal function (Go *et al.*, 2018). The hippocampus is involved in emotional processes and volumetric reductions in the hippocampus are extensively reported in major depressive disorders (MacQueen *et al.*, 2008; Fonseka *et al.*, 2018). Stress-mediated neurotoxic processes, including enhanced inflammation and neurotransmitter disturbances, may contribute to hippocampal structural decline as the illness advances (Kim and Won, 2017; Belleau *et al.*, 2019). Piperlongumine could also induce apoptosis and autophagy through targeting the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) and p38 signaling pathways (Shrivastava *et al.*, 2014; Wang *et al.*, 2018), and these signaling pathways are involved in the pathophysiology of depression

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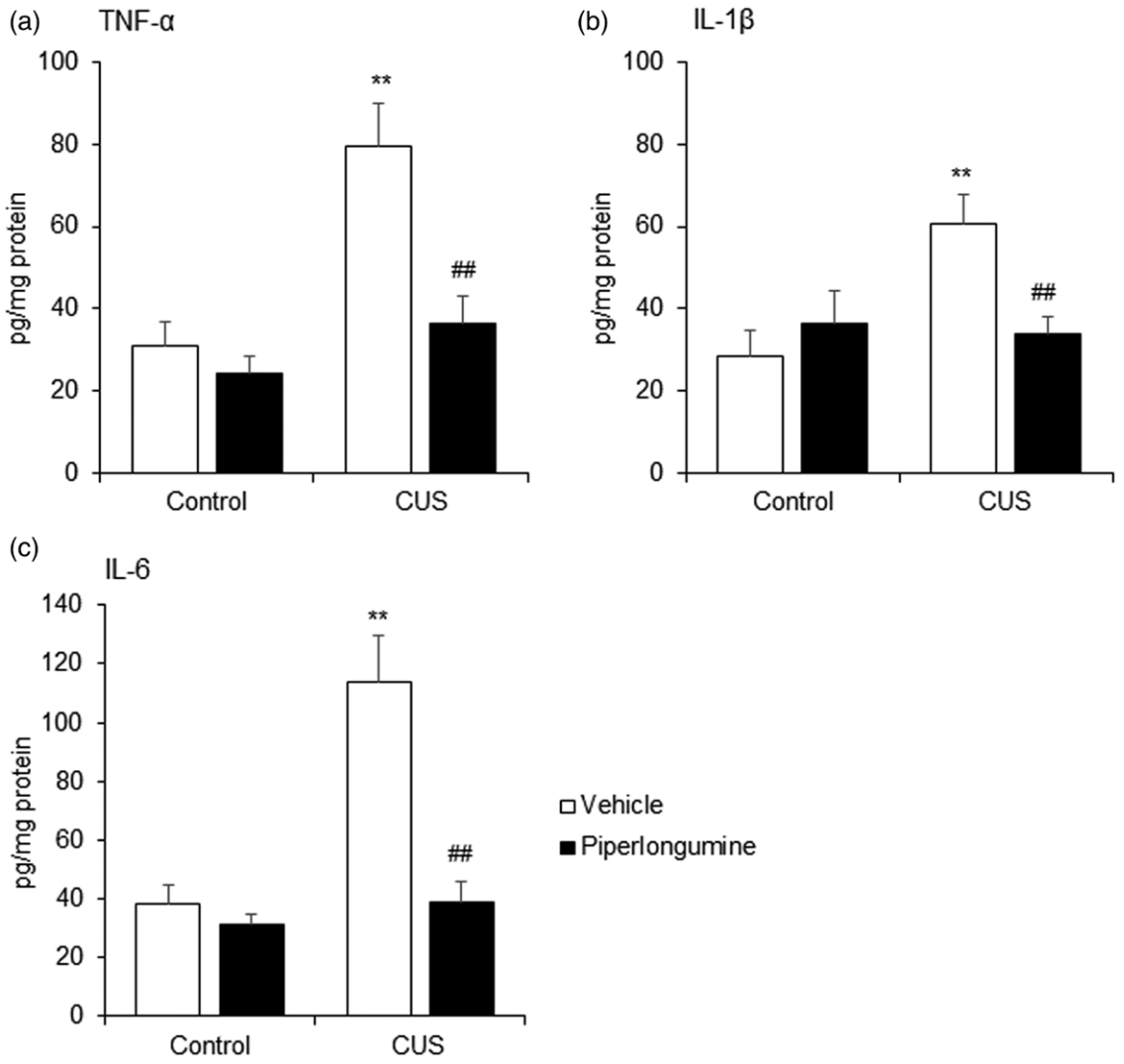
Fig. 4



Piperlongumine suppressed the expression of proinflammatory cytokines in the hippocampus induced by CUS. Oral administration of piperlongumine (25 mg/kg) for 4 weeks decreased the protein levels of TNF-α (a), IL-1β (b) and IL-6 (c) in the hippocampus of rats exposed to CUS. $^{**}P<0.01$ compared with the Control-vehicle group. $^{\#\#}P<0.01$ compared with the CUS-vehicle group. CUS, chronic unpredictable stress; IL, interleukin; TNF-α; tumor necrosis factor-α.

and action of antidepressants (Shi *et al.*, 2012; Zhao *et al.*, 2018). Consistent with previous studies (Dong *et al.*, 2018; Finnell and Wood, 2018), we found that the proinflammatory cytokines TNF-α, IL-1β and IL-6 were increased in the hippocampus of rats exposed to CUS, which could be reversed by chronic piperlongumine treatment. The anti-inflammatory state in the hippocampus may represent a mechanism of action of piperlongumine treatment.

Hypothalamic–pituitary–adrenal axis hyperactivity, reflected by elevations in cortisol, and hippocampal neurogenesis dysfunction play important roles in the etiology and treatment of depression (Pariante and Lightman, 2008; Eisch and Petrik, 2012; Fischer *et al.*, 2017). Previous studies showed that piperlongumine treatment decreased cortisol levels under stress condition (Yadav *et al.*, 2015), and increased neurogenesis in the dentate gyrus of the hippocampus (Go *et al.*, 2018), which could also contribute to its antidepressant effects.

In conclusion, oral piperlongumine administration exerted significant antidepressant-like effects in the FST and CUS, which were associated with normalization of proinflammatory cytokines levels under conditions of chronic stress. This study provides evidence for the development of piperlongumine as a promising antidepressant agent. Further research is needed to determine whether the antidepressant effects of piperlongumine in rodents are applicable to depressed patients.

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Conflicts of interest

There are no conflicts of interest.

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