Regular Article

SuHeXiang Essential Oil Inhalation Produces Antidepressant- and Anxiolytic-Like Effects in Adult Mice

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SuHeXiang (SHX) has been used to treat a wide range of diseases, including those related to the central nervous system. However, the effects of SHX on mood disorders are still elusive. This study aimed to investigate the effects of SHX essential oil on stress-induced depression of mice. In an acute stress-induced depression model, mice inhaled vehicle (1% Tween 80) for 10 min or 10% SHX for 10 or 30 min once daily for 12 continuous days. In the chronic mild stress (CMS)-induced depression model, mice were exposed to a 28-d CMS treatment. Tail suspension test (TST), forced swimming test (FST), sucrose preference test (SPT), open field test (OFT), and novelty suppressed feeding (NSF) test were conducted. In addition, serum levels of angiogenin (ANG), thrombopoietin (TPO), interleukin 6 (IL-6), and tumor necrosis factor-*α* (TNF-*α*) were evaluated by enzyme-linked immunosorbent assay (ELISA) assays. The results showed that in mice exposed to acute stress, repeated SHX inhalation exerted significant antidepressant and anxiolytic activities, and also reduced the serum levels of ANG, TPO, IL-6, and TNF-*α*. It also significantly reversed the depressive and anxiety-like behaviors, and reduced the serum levels of ANG and TPO in mice exposed to CMS. This is the first report to show that SHX inhalation could produce significant antidepressant and anxiolytic-like effects. These effects might be mediated by SHX ability to modulate the inflammatory response, and reduce dysfunction of vascular genesis and thrombosis. These results support further exploration for developing SHX inhalation as a novel therapeutic strategy for depression and stress-related disorders.

Key words SuHeXiang; stress; depression; anxiety; angiogenin; thrombopoietin

Depression, one of the most devastating mental illnesses with substantial morbidity and high suicide rate, contributes to the global burden of disease resulting in serious consequences.[1,2] The main problem in treating depression is that most of the antidepressants used in the clinic have significant limitations. For example, slow onset of action, low response rates, and even the development of drug resistance.[3] The interaction between genes and environment is one of the key factors in the pathology of depression. Substantial evidence from animals and humans show that hyperactivation of the hypothalamic–pituitary–adrenal (HPA) axis and increased inflammation, significantly damages blood vessels and even expedites thrombosis in individuals exposed to stress.[4] Normal activation of the HPA axis positively affects brain function, which ensures a proper behavioral response to the stressor,[5] while dysfunction of the HPA axis contributes to the development of depression.[6] Continuous activation of the central and/or peripheral immune system may also promote the development of major depression, and the use of anti-inflammatory therapeutics were reported to exert antidepressant-like effects.[7–9] Increasing evidence indicated that antidepressants can increase angiogenesis of the hippocampus in humans. This indicates the potential role of angiogenesis in the recovery from depressive disorders, and hints at the possibility that neurogenesis is an important response to angiogenesis.[4,10,11] As expected, vascular injury induces neurodegeneration,[12] accordingly, platelet aggregation and thrombus are an important response to stress and depression.[13,14]

Considering the potential significant neuroprotective effects of natural products, more attention is being given to discovering new antidepressants, with a focus on the active constituents, or the natural products themselves.[15] Lavender, l-perillaldehyde, musk, and hydrogen gas (see recent publication), were reported to produce antidepressant and anxiolytic-like behaviors in animals.[16–18] Chen and colleagues reported that the Yueju pill exerts rapid antidepressant-like effects both in rodents and in human.[19–21] Many kinds of constituents extracted from natural products, such as sulforaphane, and cipadesin A, could produce significant neuroprotective and antidepressant-like effects.[22,23] SuHeXiangWan (SHXW), a famous Chinese traditional medicine, is widely used to treat seizures, infantile convulsions, sudden loss of consciousness, and stroke.[24] Recent reports showed that SHXW administration exerts significant neuroprotective effects in Alzheimer's disease (AD) in drosophila and mouse models.[25–27] Essential oil from SHXW or SHXW itself could attenuate the memory impairment both in a transgenic mouse model of AD, and in amyloid-treated mice. Essential oil from SHXW also exerts regulatory effects on the central nervous system (CNS) *via* the *γ*-aminobutyrate (GABA)ergic system.[28] SuHeXiang (SHX), extracted from the trunk resin of *Lipuidambarorientalis* Mill, often acts as one of the active constituents of SHXW.

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The potential effects of SHX administration on the CNS are still elusive. In the present study, using a mouse model of stress-induced depression, the potential antidepressant and anxiolytic-like effects of SHX essential oil, and the associated mechanisms were investigated.

MATERIALS AND METHODS

Animals Male ICR mice (aged 7 weeks old upon arrival) were housed in groups with free access to food and water. The animals were maintained at a constant temperature (23±2°C) with 12 h/12 h (light on at 8 : 00 p.m., off at 8 : 00 a.m.) light/dark cycles. All animal experiments were conducted according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Local Committee on Animal Care and Use and Protection of the Hebei Medical University.

Drugs SHX essential oil was diluted in 1% Tween 80 (diluted in distilled water) to obtain concentrations of 10% (v/v). The 10% SHX essential oil was inhaled 10 or 30 min once daily for a total of 12 d. Mice in vehicle group inhaled 1% Tween 80 for 10 min once daily.

Inhalation Apparatus The inhalation apparatus were plastic boxes with lids. There were two holes (the diameter was about 1.5 cm) on the boxes for the mice breathing. Two cotton balls soaked in 2 mL of 10% SHX essential oil or 1% Tween 80, were placed in spherical gridding containers (the diameter was 5 cm) during SHX or vehicle (VEH) inhalation. When the mice were put in the boxes their lids could be closed. Every cotton ball was used only once. After the inhalation time was reached, the boxes were scrubbed with 75% alcohol.

Chronic Mild Stress The chronic mild stress (CMS) protocol was adapted from previous studies.[22,29] Briefly, mice were exposed to different unpredictable stressors for 28 d total. The stressors included restraint for 2 h, forced cold swimming for 5 min, water deprivation for 24 h, food deprivation for 12 h, tilted cages for 24 h, soiled bedding for 24 h, light/dark cycle reversal for 24 h, tail clamp for 1 min, crowding for 24 h, and bedding deprivation for 24 h. Two of ten mild stressors were randomly provided every day and performed in a variable sequence. Mice in the naïve group were kept in homepage in their housing room without any stress.

Open Field Test The open field test (OFT) was based on previous research.[22,23] The apparatus consisted of a (40×40×35 cm) square arena. Mice were individually placed in the center of the apparatus. The test session was videotaped and was analyzed by a video tracking system (SMART 3.0, Panlab, Spain). The total distance and the time spent in the central zone of mice during the 5-min test were recorded to reflect the horizontal locomotion activity and the anxiety-like behaviors, respectively.

Novelty-Suppressed Feeding Test Novelty-suppressed feeding (NSF) was done according to our previous reports.[23,30] The apparatus was a (40×40×35 cm) square arena. Before the test, all mice were deprived of food for 24 h. Mice were individually placed in the corner of apparatus with a small pellet of food on the center of the floor. After the mice ate the food, they were transferred to their home cages. The latency to feeding (in seconds, maximum time, 300 s), and food consumption during the 10 min in home cage, was measured to assess the anxiety-like behaviors and the appetite of mice.

Sucrose Preference Test Sucrose preference test (SPT) was conducted to assess the anhedonia of mice based on previous procedure.[22] During the habituation session, two bottles of 1% sucrose solution (w/v) were provided in each cage for 48 h. During the test session, mice were deprived of water for 24 h and individually housed before being exposed to two bottles for 24 h; one bottle was filled with a 1% sucrose solution, and the other was filled with water. Sucrose and water consumption during the test session were measured to conduct the depressive-like behavior of mice. Sucrose preference (%)=sucrose consumption/(sucrose consumption+water consumption)×100.

Forced Swimming Test Forced swim test (FST) was modified from our previous reports.[21,31] A 20 cm diameter×35 cm high plastic cylinder was filled to a depth of 20 cm water (23–25°C). Mice were introduced into the water for a 6-min test session. The floating time (in seconds) in the last 4 min of the test session was recorded to reflect the depressive-like behavior of mice. The time (in seconds) from being put into the water to stopping of movement was noted in the first 2 min of the 6-min session as latency to floating (maximum time, 2 min). Floating status of mice means the absence of movement, except for motion necessary to keep the heads of the mice above the water for breathing.

Tail Suspension Test Tail suspension test (TST) was performed following the existing protocol.[31,32] Mice were fixed by adhesive tape placed approximately 1 cm from the tip of the tail and were suspended 50 cm above the floor for a 6-min test session. The total time spent immobile in the last 4 min was recorded to reflect the depressive-like behavior. The latency time to being immobile (in seconds, maximum time, 120 s) was also recorded. Immobility was defined as the stillness of limb and body, except for those movements needed to respire. During the test, mice were separated from each other to prevent possible visual and acoustical associations.

Enzyme-Linked Immune-Sorbent Assay (ELISA) ELISA was done according to the previous report.[22] In brief, about a 1 mL blood sample was collected from one mouse through extracting from the eyeballs after the last behavioral test. Blood samples were placed at room temperature for 20 min and were centrifuged at 2000 rpm for 20 min. The serum was transferred into new tubes for ELISA analyses. The measurement of serum levels of angiogenin (ANG), thrombopoietin (TPO), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α) was conducted with commercially available ELISA kits (ANG, ml650713; TPO, ml651370; IL-6 ml002293; TNF-α, ml002095; Mlbio, China).

Experimental Design

Experiment 1

Effects of repeated SHX inhalation on depressive- and anxiety-like behaviors in mice exposed to acute stress

As shown in Fig. 1A, experiment 1 was aimed to determine the antidepressant and anxiolytic-like effects of repeated SHX inhalation and the associated underlying mechanism (*i.e.*, angiogenesis- and inflammation-related molecules), in mice exposed to acute stress. After a 6-d habituation, thirty mice were randomly divided into 3 groups (n=10 per group). Mice in vehicle group (VEH group) inhaled 1% Tween 80

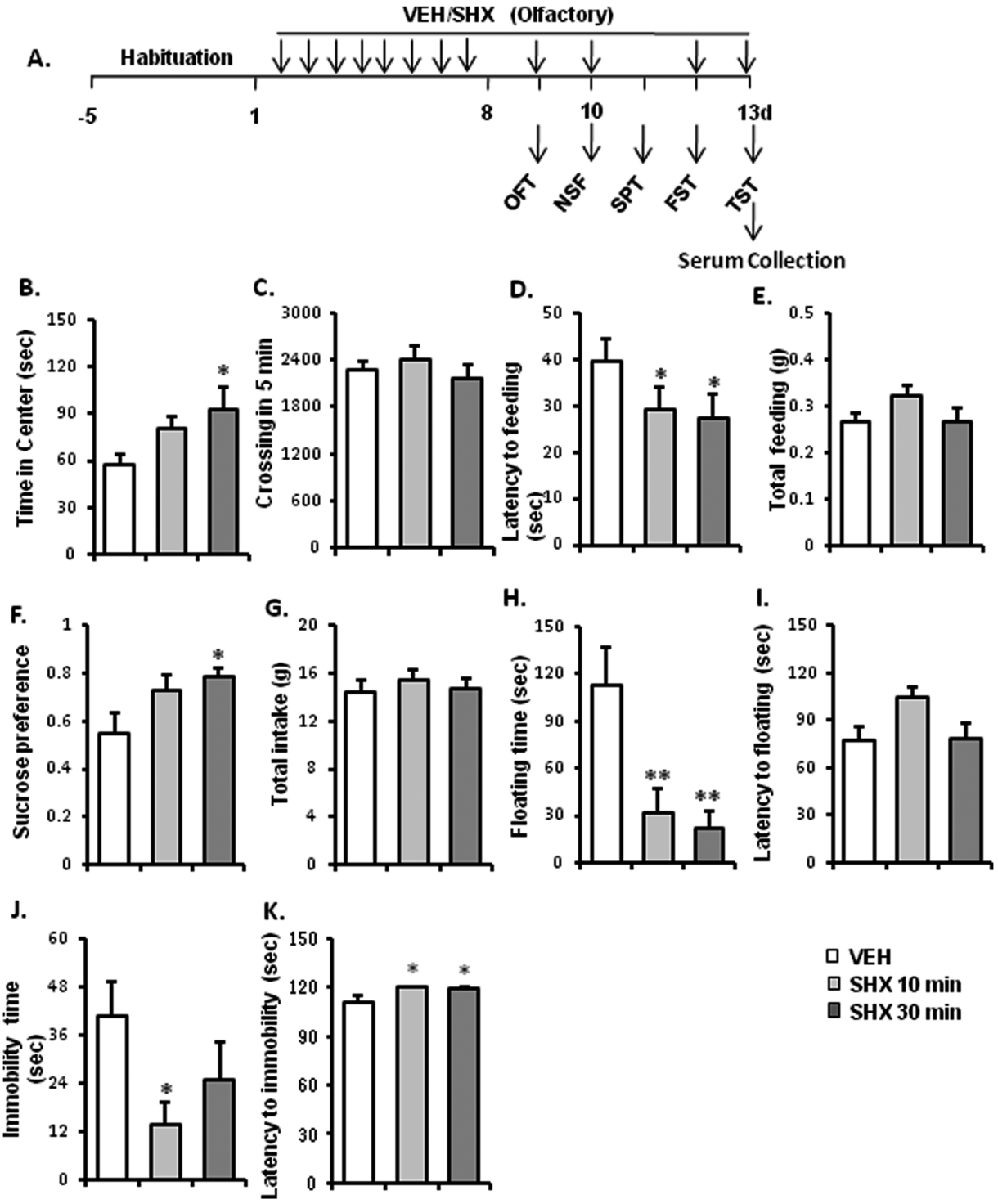

Fig. 1. SHX Inhalation Exerted Antidepressant- and Anxiolytic-Like Effects in Mice Exposed to Acute Stress

(A) Experimental procedure. After a 6-d adaptation period, the mice were exposed to 1% Tween 80 for 10 min, 10% SHX for 10 min or 30 min daily for 12 d. From the 9th day, behavioral tests were conducted. SHX inhalation significantly increased time spent in the central zone (B) without affecting the crossing activities (C) in the OFT, decreased latency to feeding (D) without affecting the total feeding (E) in the NSF, increased sucrose preference (F) without affecting the total intake (G) in the SPT, reduced floating time in the FST (H) without effects on the latency to floating (I), decreased the immobility time in the TST (J) and prolonged latency to immobility (K). $*p<0.05$, $**p<0.01$ *versus* the VEH group. $n=6-10$ per group.

for 10 min. The other two groups of mice were treated with inhalation of 10% SHX for 10 min (SHX10 group) or 30 min (SHX30 group) once daily for 12 continuous days. On the ninth day, behavioral tests mainly including OFT, NSF, SPT, FST, and TST, were conducted according to the experimental procedure. All behavioral tests were conducted 30 min after the intraday inhalation. Blood samples were collected for further ELISA analyses after the TST tests.

Experiment 2

Effects of repeated SHX inhalation on depressive and anxiety-like behaviors in CMS-treated mice

As shown in Fig. 3A, the antidepressant- and anxiolytic effects of SHX inhalation from chronic mild stress (CMS), and the associated mechanism mediating the effects of SHX

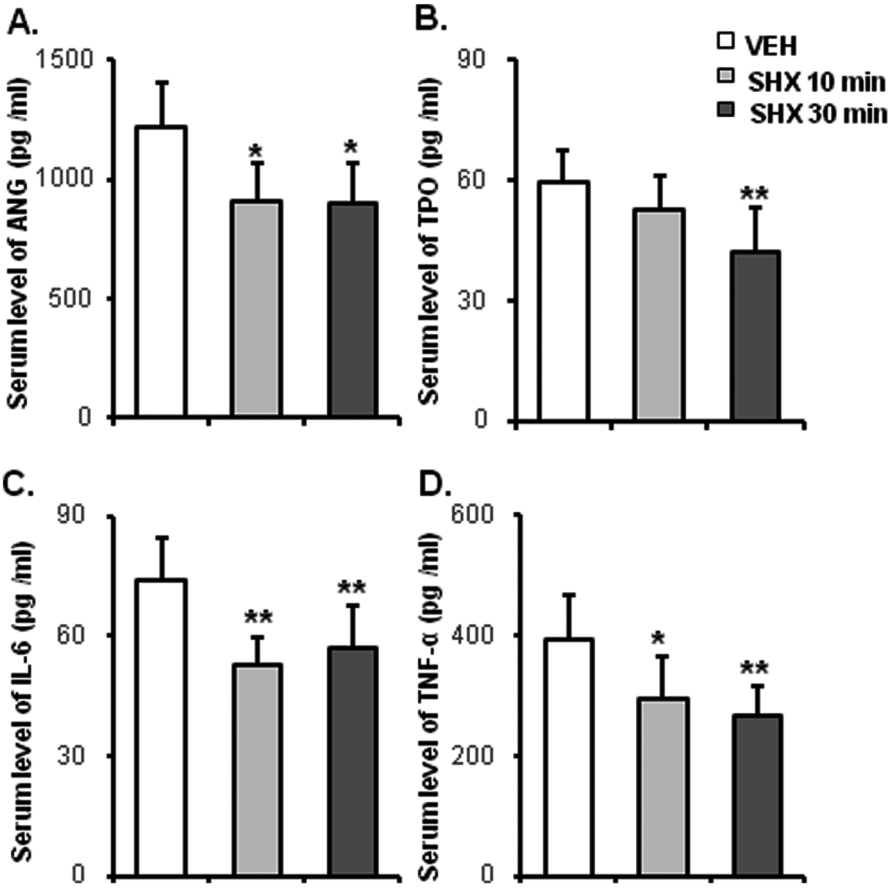

Fig. 2. SHX Inhalation Decreased the Serum Levels of ANG, TPO, IL-6 and TNF-α in Mice Exposed to Acute Stress

Blood samples of mice were collected for ELISA analysis after the last behavioral tests in the acute stress experiment. SHX inhalation significantly reduced the serum levels of ANG (A), TPO (B), IL-6 (C) and TNF-α (D). *$p<0.05$, **$p<0.01$ *versus* the VEH group. $n=4$–6 per group.

inhalation were investigated. After a 5-d habituation, forty mice were divided into 2 groups, naïve group ($n=10$) and CMS group ($n=30$) based on the baseline results of SPT and NSF. Mice in CMS group were exposed to a 28-d CMS course and then were randomly divided into 3 subgroups for inhaling with 1% Tween 80 for 10 min (CMS+VEH group), 10% SHX for 10 min (CMS+SHX10 group) and 30 min (CMS+SHX30 group). SHX inhalation was performed once daily for 12 continuous days. Mice in naïve group were kept in their home cages without any treatment. After a consecutive 8-d inhalation, behavioral tests, including OFT, NSF, SPT, FST and TST were validated 30 min after the intraday inhalation. Blood samples were collected for ELISA analyses after the last behavior tests.

Data Analysis All data were described as mean±standard error of the mean (S.E.M.) Statistical analysis between two groups used independent *t*-test. The statistical analysis among multi-groups, used one-way ANOVA. The significant level was defined as $p<0.05$.

RESULTS

Repeated SHX Inhalation Produced Antidepressant- and Anxiolytic-Like Effects in Mice Exposed to Acute Stress To validate the antidepressant and anxiolytic-like effects of SHX inhalation on mice exposed to acute stress, mice were randomly divided into 3 groups ($n=10$ per group): VEH group, SHX 10 min group, SHX 30 min group, as mentioned above. Inhalation was performed once daily and repeated 12 d. Behavioral tests were operated 30 min after the intraday inhalation.

One-way ANOVA of the OFT and NSF data revealed a significant effect of SHX inhalation on anxiety-like behaviors. Data analyses showed that repeated SHX inhalation for 30 min significantly increased the time spent in the central zone in the OFT ($F_{2,21}=3.805$, $p<0.05$, Fig. 1B) compared with that of mice in VEH group. Repeated SHX inhalation at all durations had no significant effects on the crossing activities in the OFT ($F_{2,21}=0.633$, $p>0.05$, Fig. 1C), indicating that the effects of SHX inhalation on the time spent in the central zone was not caused by affecting the locomotion activities of mice. Results from the NSF showed that repeated SHX inhalation significantly decreased the latency to feeding ($F_{2,23}=4.108$, $p<0.05$, Fig. 1D), without any effects of total feeding in this test ($F_{2,23}=1.966$, $p>0.05$, Fig. 1E) compared with that of mice in VEH group.

The effects of repeated SHX inhalation on depressive-like behaviors were conducted by SPT, FST and TST. One-way ANOVA of the data from SPT, FST and TST revealed a significant antidepressant-like effect of SHX inhalation. Repeated SHX inhalation for 30 min ($F_{2,22}=4.019$, $p<0.05$, Fig. 1F), but not 10 min ($F_{2,22}=4.019$, $p>0.05$, Fig. 1F) daily significantly increased the sucrose preference compared with that of mice in VEH group, without any significant effects on the total water intake of mice ($F_{2,22}=0.657$, $p>0.05$, Fig. 1G). SHX inhalation significantly reduced floating time ($F_{2,23}=11.203$,

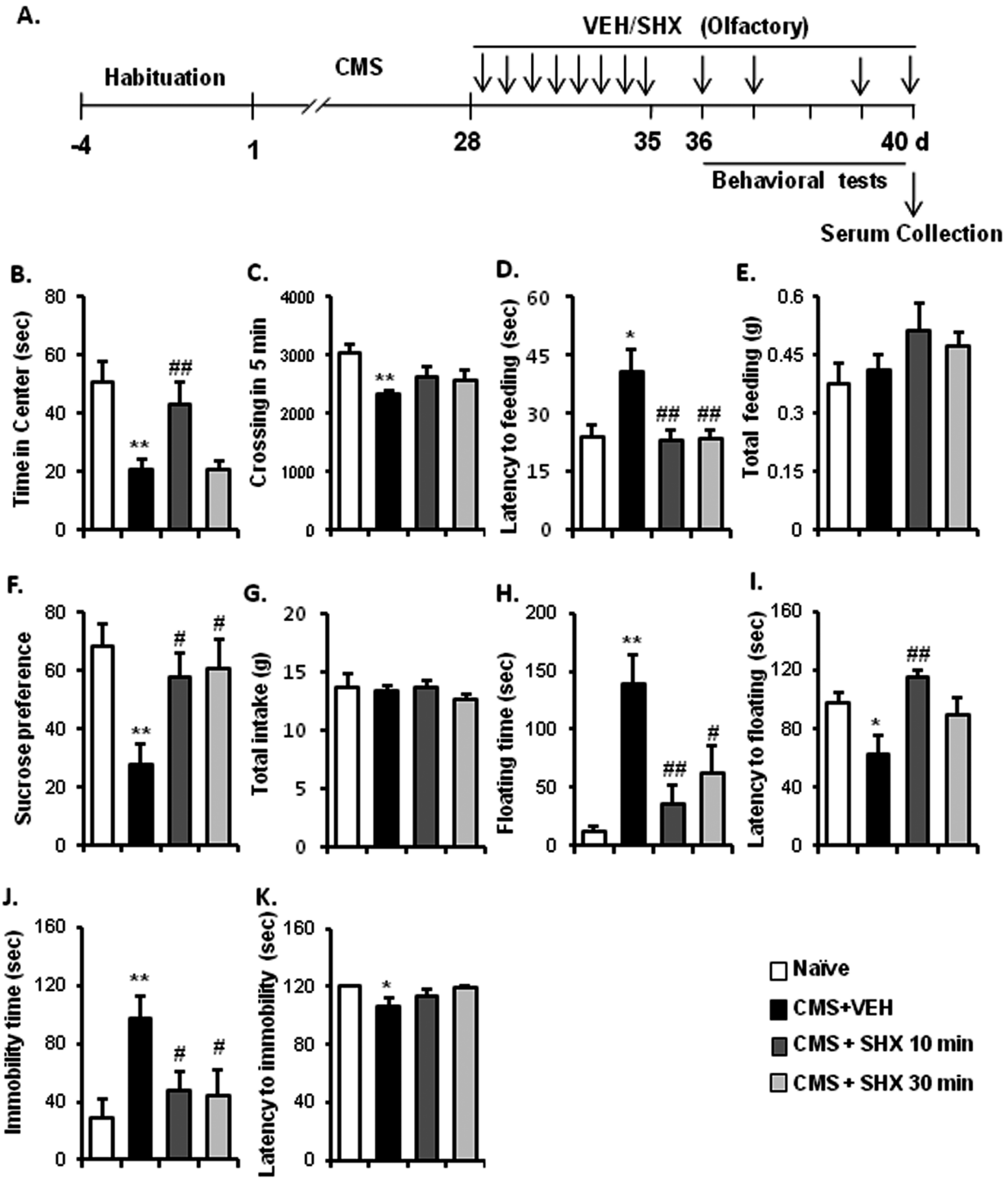

Fig. 3. SHX Inhalation Blocked CMS-Induced Depressive- and Anxiety-Like Behaviors in Mice

(A) Experimental procedure. After a 5-d adaptation period, mice were treated by chronic stress for 28 d. On day 28, mice were exposed to 1% Tween 80 for 10 min, 10% SHX for 10 or 30 min for 12 d. During day 36–40, behavioral tests were conducted. SHX inhalation significantly blocked the decreased time spending in the central zone (B) without affecting the crossing activities (C) in the OFT, the prolonged latency time to feeding (D) without affecting the total feeding (E) in the NSF, the decreased sucrose preference (F) without affecting the total intake (G) in the SPT, the prolonged floating time (H) and shortened latency to floating (I) in the FST and the prolonged immobility time (J) without affecting the latency to immobility (K) in the TST. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* CMS+VEH Group; $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Naïve Group. n=7–8 per group.

$p<0.01$, Fig. 1H) without affecting on the latency to floating of mice in the FST group ($F_{2,\,23}=2.709$, $p>0.05$, Fig. 1I). SHX inhalation reduced the immobility time ($F_{2,\,19}=2.951$) and Post Hoc Multiple Comparisons indicated that SHX inhalation for 10 min ($p<0.05$, Fig. 1J), but not for 30 min ($p>0.05$, Fig. 1J) significantly reduced the immobility time. SHX inhalation also prolonged the latency to immobility ($F_{2,\,19}=4.223$, $p<0.05$, Fig. 1K) compared with that of mice in VEH group in the TST. Data from these results indicate that SHX inhalation produces antidepressant- and anxiolytic-like effects in mice in response to acute stress.

Repeated SHX Inhalation Decreased the Serum Levels of ANG, TPO, IL-6 and TNF-α in Mice Exposed to Acute Stress To explore the possible mechanism mediating the

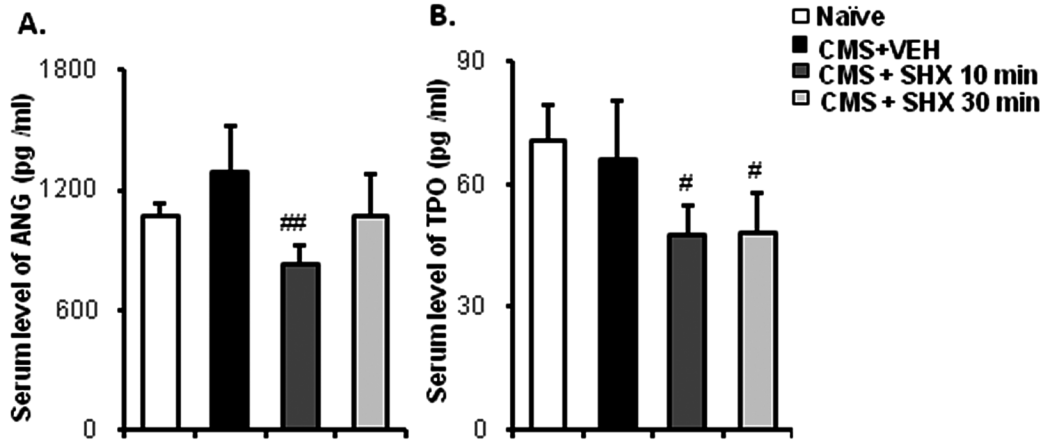

Fig. 4. SHX Inhalation Decreased the Serum Levels of ANG and TPO in CMS-Treated Mice

Blood samples of mice were collected for ELISA analysis after the last behavioral tests in the CMS procedure. SHX inhalation reversed the increased serum ANG (A), further decreased serum TPO (B). $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* CMS+VEH Group; $n=4–6$ per group.

antidepressant and anxiolytic activities of SHX inhalation, serum sample were collected after the last behavioral test for ELISA analyses. One-way ANOVA analysis showed that significant effects of SHX inhalation on serum ANG, TPO, TNF-α and IL-6. Repeated SHX inhalation significantly decreased the serum level of ANG ($F_{2,\ 11}=5.118$, $p<0.05$, Fig. 2A), TPO ($F_{2,\ 13}=4.824$, $p<0.01$, Fig. 2B), IL-6 ($F_{2,\ 15}=8.307$, $p<0.01$, Fig. 2C) and TNF-α ($F_{2,\ 15}=6.291$, $p<0.05$; $p<0.01$; Fig. 2D).

Repeated SHX Inhalation Blocked the Depressive- and Anxiety-Like Behaviors in CMS-Treated Mice To further investigate the effects of repeated SHX inhalation on depressive- and anxiety-like behaviors, a 28-d CMS mouse procedure was conducted. The results showed that mice subjected to CMS (CMS+VEH group) exerted significant depressive- and anxiety-like phenotype, including decreased time spent in center zone ($t_{14}=3.609$, $p<0.01$, Fig. 3B), decreased crossing activity ($t_{14}=3.940$, $p<0.01$, Fig. 3C) in the OFT, and prolonged latency to feeding ($t_{13}=-2.646$, $p<0.05$, Fig. 3D) inthe NSF compared with that of mice in naïve group. Repeated SHX inhalation significantly reversed the anxiety-like behaviors of mice exposed to CMS, including increased time spent in center zone ($F_{2,\ 20}=7.674$, $p<0.01$, Fig. 3B) in the OFT, decreased latency time to feeding ($F_{2,\ 20}=6.716$, $p<0.01$, Fig. 3D) in the NSF compared with that of mice in CMS+VEH group. No significant effects on crossing activity ($F_{2,\ 20}=1.080$, $p>0.05$, Fig. 3C) and total feeding ($F_{2,\ 20}=0.882$, $p>0.05$, Fig. 3E) were found within these three CMS groups.

The *t*-test analysis showed that CMS induced significant depressive-like phenotypes, reflected by a decreased sucrose preference ($t_{14}=3.977$, $p<0.01$, Fig. 3F), an increased floating time ($t_{14}=-4.988$, $p<0.01$, Fig. 3H), shortened latency to floating ($t_{14}=2.368$, $p<0.05$, Fig. 3I), prolonging immobility time ($t_{14}=-3.325$, $p<0.01$, Fig. 3J), and shortening latency to immobility ($t_{14}=2.260$, $p<0.05$, Fig. 3K). One-way ANOVA analysis showed that SHX inhalation significantly increased the sucrose preference ($F_{2,\ 20}=4.605$, $p<0.05$, Fig. 3F), decreased the floating time ($F_{2,\ 20}=6.387$, $p<0.01$; $F_{2,\ 20}=6.387$, $p<0.05$; Fig. 3H), and shortened the immobility time ($F_{2,\ 20}=3.665$, $p<0.05$, Fig. 3J) compared with that of mice in CMS+VEH group. Further, mice with 10-min SHX inhalation exerted significantly increased latency time to floating ($F_{2,\ 20}=7.265$, $p<0.01$, Fig. 3I) compared with mice in CMS+VEH group. No significant effects were observed on total intake ($F_{2,\ 20}=1.129$, $p>0.05$, Fig. 3G) and latency time to immobility ($F_{2,\ 20}=1.820$, $p>0.05$, Fig. 3K) among mice exposed to CMS. Above all, results indicate that SHX inhalation exerted significant antidepressant- and anxiolytic-like effects in chronically stressed mice.

Repeated SHX Inhalation Reversed the Serum Levels of ANG and TPO in CMS Mice ELISA analyses showed that the 28-d CMS induced the tendency of elevated serum ANG and decreased serum TPO. SHX inhalation for 10 min significantly reversed the increase of ANG ($F_{2,\ 12}=6.580$, $p<0.01$, Fig. 4A), and decreased the TPO serum concentration($F_{2,\ 12}=4.662$, $p<0.05$, Fig. 4B). SHX inhalation for 30 min also significantly decreased the serum concentration of TPO ($F_{2,\ 12}=4.662$, $p<0.05$, Fig. 4B).

DISCUSSION

SHXW, acting as a traditional Chinese medicine, has been widely used orally for the treatment of seizures, stroke and infantile convulsions. Recently, SHXW and its essential oil were reported to alleviate amyloid beta-induced neurodegenerative abnormality, such as memory impairment, *via* suppression of the hyperactivation of c-Jun N-terminal kinase (JNK) activity, glia proliferation and apoptosis in drosophila and mice models. As one of the main components of SHXW, the potential effects of SHX on depression is poorly understood. In the present study, using acute and chronic stress depression mice models, we investigated the potential depressive- and anxiety-like effects of SHX essential oil inhalation on adult mice. The results showed that repeated SHX inhalation produced significant antidepressant- and anxiolytic-like effects in mice exposed to both acute and chronic stress. Further, SHX inhalation exerted significant effects on the serum levels of ANG, TPO, IL-6, and TNF-α of mice exposed to stress, indicating the neuroprotective effects of SHX inhalation on depression and anxiety may be, at least in part mediated by its regulatory effects on the inflammatory response and/or blood vessel change.

Acute stress mice paradigms and chronic mild stress (CMS) mice model are widely used to investigate the potential antidepressant-like activities of drugs.[33] CMS could induce

significant depressive- and anxiety-like phenotypes in rodents, including anhedonia, despair, and anxiety behaviors, which could be reversed by classic and/or novel potential antidepressants.[34–36] The FST and TST models are widely used for assessing despaired behaviors and the putative antidepressant-like activities of compounds in mice exposed to both acute and chronic stress.[23,29,37] The immobility time in the TST and floating time in the FST negatively reflect the depressive-like status. SPT was used to assess the anhedonia of depressed animals, the lower sucrose preference, and the higher level of depression of animals. In our present study, we found that repeated SHX essential oil inhalation significantly decreased the floating time in the FST, decreased the immobility time in the TST, decreased the latency to feeding in the NSF, while increased the time spent in the central zone in the OFT of mice exposed to acute stress. Results from the CMS-induced depression mice showed that repeated SHX inhalation significantly blocked the CMS-induced depressive- and anxiety-like behaviors in mice exposed to CMS, which further confirms the antidepressant and anxiolytic-like effects of SHX inhalation.

In addition to those classic theories, including hyperactivation of HPA axis, dysfunction of neurogenesis, dysregulation of inflammation and oxidative stress injuries,[38] neuroimmune mechanism of depression has attracted more and more attention.[39] Anti-inflammatory therapeutics could significantly reverse these levels in the related brain regions and in the serum of both rodents and human.[40,41] Our results show that IL-6 and TNF-α, two well-investigated pro-inflammatory cytokines in depression, significantly decreased after repeated inhalation of SHX essential oil. An improvement in the depressive- and anxiety-like behaviors in mice exposed to acute stress was also observed, which indicates that the enhanced adaptability to stress from SHX inhalation may be associated with its regulation of the peripheral immune system in mice.

Stress, especially chronic stress could induce dysregulation of immune response and subsequent vascular pathology.[42] Chronic inflammation and sustained increases in circulating pro-inflammatory cytokines have been associated with atherosclerotic plaque formation, progression and can diffuse into the brain of stressed individuals as a result of stress-induced neurovascular damage and increased blood–brain barrier (BBB) permeability facilitate the development of depressive-like behavior.[42] Among these, angiogenesis and platelet activities are associated with the occurrence and development of depression.[10,43,44] Antidepressant therapies could increase angiogenesis, and neurogenesis occurs in the region of active angiogenesis.[45,46] Increased platelet aggregation and a higher risk of thrombus are also reported to be associated with depression.[13,14] ANG, a potent stimulator of new blood vessels, could promote angiogenesis under hypoxia, inflammation, and in the acute phase response.[47,48] Higher serum levels of ANG were reported in nurses with high levels of stress.[49] Our ELISA data showed that SHX inhalation significantly decreased the serum level of ANG in mice in response to acute stress. The 28-d CMS procedure induced a tendency to increase the serum level of ANG, which was significantly reversed by repeated SHX inhalation. This highlights the possibility that the antidepressant-like effects of SHX inhalation may also be mediated by strengthening the blood vessels and inducing angiogenesis in mice exposed to stress. Acute stress would result in hypercoagulability and even thrombus, and that the susceptibility to chronic social stress increases plaque progression and platelet activation.[50–53] Unexpectedly, SHX inhalation significantly decreased the serum level of TPO, which is an important molecule associated with platelet activity in mice exposed to acute stress.[54] Although no significant change was observed in mice exposed to CMS, repeated SHX inhalation significantly decreased the serum level of TPO, accompanied with the antidepressant and anxiolytic-like effects in mice exposed to CMS. This is consistent with the previous report[55] that SHXW prevented the collection of platelet in cerebrum, and it was effective against the formation of thrombus. Thrombosis might participate in the pathological process of depression and anxiety caused by stress but SHX prevented it.

In summary, our data for the first time, to our knowledge, revealed that SHX essential oil inhalation exerts significant antidepressant- and anxiolytic-like effects in mice. The antidepressant- and anxiolytic-like effects of SHX essential oil inhalation may be associated, at least in part, with its potential regulatory functions on inflammatory response and/or thrombosis. However, limitations are still exist in the present study. First, considering the important role of the olfactory system in the development of depression and antidepressant therapy and the treatment style of SHX essential oil inhalation, whether the olfactory system is directly involved in the antidepressant-like effects of SHX essential oil inhalation and what is the downstream underlying mechanism. Secondly, compared with SHXW, there are fewer components in essential oil of SHX, identification of the mainly effective components will be very important. Next, dose- and time dependent effects of SHX essential oil inhalation should be investigated. Last, since the high safety and convenience of SHX, the possible application of SHX essential oil for treatment of patients with depression and other stress-related disorders should be carried out for further investigated.

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Conflict of Interest The authors declare no conflict of interest.

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