### Schisandrin B alleviates acute oxidative stress via modulating Nrf2/Keap1-mediated antioxidant pathway

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Schisandrin B alleviates acute oxidative stress via modulating Nrf2/Keap1-mediated antioxidant pathway

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Abstract: Schisandrin B (Sch B), one of Fructus Schisandrae’s main effective components, protects neurons from oxidative stress in the central nervous system. Here we investigated the neuroprotective effect of Sch B in the acute oxidative stress damage and attempted to define the possible mechanisms. From the elevated plus maze (EPM) and open field test (OFT), we found that forcing swimming, an acute stressor, significantly induced anxiety-like behavior which was alleviated by Sch B (p.o.) treatment. In addition, the Sch B treatment suppressed toxicity, malondialdehyde (MDA) and reactive oxygen species (ROS), an important factor for neuron damage. The antioxidant molecules under the control of Nrf2 pathway, such as superoxide dismutase (SOD) and glutathione (GSH), were significantly increased by Sch B treatment. Moreover, a higher percentage of intact cells in the amygdala further verified the neuroprotective effect of Sch B in Nissl staining. Several proteins such as Nrf2 and its endogenous inhibitor Keap1, were abnormal expressed in force swimming mice but were significantly reversed by Sch B treatment. Herein, our results suggested that Sch B may be a potential therapeutic agent against anxiety disease that is associated with oxidative stress. The possible mechanism is attributed to its neuroprotection through enhancing antioxidant effect.

Key words: Schisandrin B; Force swimming; Oxidative stress system; Nrf2/Keap1 pathway;

Abbreviations: Sch B, Schisandrin B; OFT, open field test; EPM, elevated plus maze; SOD, superoxide dismutase; ROS, reactive oxygen species; MDA, malondialdehyde; GSH, glutathione; Keap1, Kelch-like erythroid cell-derived protein 1;
Introduction

Oxidative stress, which is commonly described as an imbalance between the reactive oxygen species (ROS) generated and clearance by the endogenous antioxidant defense system, represents common denominators for the central nervous system (CNS) diseases (Hovatta et al. 2010). Acute or chronic exposure to stress evokes a cascade of physiological reactions accompanied with changed behavioral state, such as anxiety-like behaviors, a common category of psychiatric disorders characterized by nervous and anxious feelings in modern society (Alonso and Lepine 2007, Ulrich-Lai et al. 2015). Some research reported that melatonin protected neurons from oxidative stress in sleep deprivation-associated anxiety rats model (Wei et al. 2015). The deletion of an anti-oxidative enzyme, glutathione peroxidase 4 (Gpx4) from dopaminergic neurons induced anxiety-like behavior (Schriever et al. 2017). However, it remains worth exploring that how to alleviate stress induced anxiety behaviors via reducing oxidative stress Schisandrin B (Sch B), a dibenzocyclooctadiene derivative isolated from the fruit of Schisandra chinensis (Turcz.) Baill (Zhang et al. 2015), has obviously clinical relevance as hepatoprotective, anti-inflammatory, anti-cancer, anti-oxidantive effect (Ip and Ko 1996, Wang et al. 2007, Lin et al. 2017, Sun et al. 2017). Recent experimental findings have further demonstrated that Sch B protects neurons from oxidative stress by eliminating oxygen free radicals in CNS (Ba et al. 2015, Giridharan et al. 2015). Sch B attenuates cisplatin-induced neurotoxicity through modulating Nrf2/antioxidant response element (ARE), signal pathway an critical transcription factor for the oxidative stress (Li et al. 2012). Nrf2 uncoupled from Keap1 (Kelch-like erythroid cell-derived protein 1) transfers into the nuclear and spurs the cascade reactions of antioxidant response elements.
Pretreatment with Sch B could reduce malondialdehyde (MDA) and ROS release, significantly increase the cell viability and the superoxide dismutase (SOD) level, which protein-encoding genes is regulated by the Nrf2 (Jiang et al. 2015). Sch B is widely applied in the Alzheimer’s disease and Parkinson's disease (Ba et al. 2015, Giridharan et al. 2015), but no data is available for its anxiolytic activity. Herein, we hypothesized that Sch B alleviates oxidative stress damage via Nrf2 pathway and further relieving the acute stress induced anxiety-like behavior.

Nrf2-mediated antioxidant response pathway is the primary cellular defense mechanism against the cytotoxic effects of oxidative stress (Ma 2013). Nrf2 pathway related to oxidative stress give us a novel understanding about the effect of Sch B on regulating the expression of Nrf2 protein in the acute oxidative stress model. Therefore, the aim of the present study is to explore the possible neuroprotective effect of Sch B by using the force swimming mice model.

We studied the behaviors of the mice with and without orally administration of Sch B, and further analyzed the underlying mechanisms that whether the anxiolytic effect is associated with the Nrf2-mediated antioxidant response pathway.

2. Materials and methods

2.1 Animals

Male C57BL/6 mice (6 weeks, 18–22 g) were obtained from the Laboratory Animal Center of the Kunming Medical University. In order to adapt the environment, animals were housed in similar cages (2-3 per cage) with food and water available ad libitum. The temperature-controlled (22±2°C) and humidity-controlled (50-60%) animal room maintained on 12-h light-dark cycle regularly. All experiments protocols involving mice were carried out...
in strict accordance with the guidelines of the Institutional Ethical Committee of the Kunming Medical University and the Guide for the Care and Use of Laboratory Animals (Washington, USA).

### 2.2 The establishment of acute stress model and drug treatment

The mice were divided randomly into four groups: the model group (stressed mice), 30mg/kg and 60 mg/kg Sch B treated group, and the control one. The stressed mice were force to swim in an open cylindrical container (diameter, 10cm; height, 30 cm) containing 20 cm of water (22± 1°C). Mice were removed from the water after force swimming 30 min for 2 consecutive days, according to previous research (Tian et al. 2013). After immediately wiped dry, they were placed into cages and given food and water ad libitum. Before force swimming, the treated mice were orally administered 30 or 60 mg/kg Sch B (dissolved in saline with 0.4% DMSO) (Jiang et al. 2015), twice daily for 3 consecutive day (Wan et al. 2017). The drug was purchased from Herbpurify Co. Ltd. (Chengdu, China, purity>98%). However, the model group was orally administered with equal saline (with 0.4% DMSO), as well as the control one. After 24h for the last force swimming, the mice were used for the following experiments.

### 2.3. Elevated plus maze (EPM)

The elevated plus maze (EPM) was carried out as described in previous reports (Walf and Frye 2007). The equipment contains two open arms (25 cm×8 cm×0.5 cm) and two closed arms (25 cm×8 cm×12 cm), with a common central platform (8 cm×8 cm). The apparatus was elevated to a height of 50 cm above the floor. Then mice were tested on the central platform, facing an open arm, and allowed to move freely for 5 min. The motor activities were recorded by camera and analyzed with a video-tracking system.
(DigBehv-EPMM, Shanghai Jiliang software technology Co. Ltd.). Meanwhile, the number of entries and time spent in open arm were the evaluation index of anxiety.

2.4. Open field test (OFT)

As described previously (Wang et al. 2017), the open field test (OFT) was conducted in an apparatus consisted of a 30cm×30cm×30cm square box (JL Behv-LAM, Shanghai, China), which the bottom is divided equally into 16 squares. Briefly, mice were tested in the center of the arena and allowed to move freely. After adapting to the environment for 2 min, the exploratory behaviors of mice were videotaped using a camera fixed above the box during a 15min session. And the central area is referring to the 4 squares in the centre, and the time in central area and travel distance were analyzed by using a motion tracking system (MedAssociates, St. Albans, VT).

2.5 The collection of serum and amygdala samples

After the last behavior test, the mice were deeply anesthetized and the blood collected from the orbit was centrifugated at 5,000 rpm for 5 min and the serum was obtained. And then the fresh amygdala tissues were dissected from each brain refer to the brain map written by George Paxinos and Keith b.j. Franklin. All the serum and amygdala samples were conserved in -80℃ for further research.

2.6 Determination of Thiobarbituric Acid- Reactive Substances

The measurement of malondialdehyde (MDA), an end product of lipid peroxidation, has been extensively used as an index of oxidative stress (Ohkawa et al. 1979). The tissue samples were homogenized in PBS at a ratio of 1:9, supernatant protein concentrations were obtained for the following research after centrifugation (12,000 rpm, 10 min). Meanwhile, serum and
supernatant protein concentrations were used to detect MDA level by using biochemical assay--TBA method (Jiancheng Bioengineering Institute, Nanjing, China). The amount of thiobarbituric acid-reactive substances (TBARS) was estimated by spectrophotometric absorbance at 532 nm (DU-800, Beckman Coulter, Fullerton, CA, USA). The absorbance measurements were calculated using a standard curve.

2.7 Measurement of Total ROS Production

The ROS level was detected by using ROS assay kit (Beyotime Institute Biotechnology, Shanghai, China). According to the fluorescence intensity, we tested the ROS level of serum and tissues through quantifying 2,7-dichlorofluorescein diacetate (DCFH-DA). The fluorescence intensity was detected using a Spectro fluorometer (F-2500, Hitachi, Tokyo, Japan) at an excitation wave length of 488 nm and an emission wave length of 535 nm.

2.8 Measurement of GSH and SOD in the amygdala and serum

Reduced glutathione (GSH) and SOD, which can eliminate ROS molecules, provide antioxidant protection (Kermanizadeh et al. 2012, Fischer et al. 2013). Therefore, depletion of GSH and decreases in SOD activity are also usually regarded as measures of oxidative stress. We detected GSH level and SOD activity of serum and amygdala tissues sample by using GSH assay kit (Spectrophotometric method) and SOD assay kit (Hydroxylamine method). All the procedures are according to specification of assay kit which brought from Jiancheng Bioengineering Institute, Nanjing, China.

2.9 Histological analyses

Histological analyses were carried out using Nissl staining for indicators of cells death in the amygdala. After the last behavior, mice were anesthetized using sodium pentobarbital (40
and perfused intracardially with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS, pH 7.4). After dehydrated, sequential coronal sections of amygdala (30-μm thickness) were acquired on HM525 freezing microtome (Microm, Germany), collected in sequence as free-floating sections in PBS. For histological assessment, every tenth section (at intervals of 300 μm) was stained with 1% cresyl violet for 30 min. After section-staining, images were collected by an Olympus BX53 microscope (Japan). The proportion of intact neurons in the amygdala was measured manually.

2.10 Western blot analysis

The amygdala samples were incubated with RIPA buffer (Cwbiotech, Beijing, China) containing a proteinase inhibitor mixture (Roche, Mannheim, Germany) and 10 μM PMSF (Sigma, USA). After ultrasonication, the samples were centrifuged at 12,000 rpm for 20 min. The supernatant was added with loading buffer (5x) and boiled in 95°C water for 10 min. Equivalent proteins were transferred to polyvinylidene difluoride membranes by using 10% SDS-PAGE gel electrophoresis. And probed with antibodies: Anti-Nrf2 (1:1000; Abcam, ab62352), Anti-Keap1 (1:500; Abcam, ab139729) and Anti-beta Actin (1:1000; Abcam, ab8227). Secondary antibodies were HRP-conjugated anti-rabbit or anti-mouse antibodies (1:10000, Cwbiotech, Beijing, China). After incubation with the appropriate HRP-coupled secondary antibody, the proteins were visualized using enhanced chemiluminescence (GE Healthcare). The density of immunoblots was conducted using ChemiDoc XRS (Bio-Rad, Hercules) and quantified using Quantity One version 4.1.0 (Bio-Rad).

2.11 Statistical Analysis

Data are expressed as mean ± SEM. Appropriate statistical approaches including one
way-ANOVA were used followed by LSD-t Test. The p value of less than 0.05 was considered statistically different. All statistical analyses were conducted using SigmaPlot 10.0 (Systat software, Point Richmond, CA, USA).

Results

1. Acute stress induced anxiety-like behaviors.

EPM and OFT were used to evaluate anxiety-like behavior of mice experiencing force swimming. The results showed that the model mice exhibited a less percentage of time in open arm (Fig. 1A) and avoided to enter the open arm in the EPM (Fig. 1B) compared with the control group. The percentage of time in central area also decreased in the OFT compared with control one (Fig. 1D). In addition, the drug did not significantly change the normal locomotor activity, as shown in Fig.1C and Fig.1E.

2. Sch B reliefs anxiety-like behavior caused by the acute stress.

The behavioral tests had confirmed that the mice experiencing force swimming is a successful anxiety model. And then we did an in-depth assessment about whether Sch B could alleviate the anxiety symptoms. To our surprised, we found that Sch B treated group, no matter which dosage, spent more time in open arm of the EPM compared with the model group (Fig. 1A). However, only 60 mg/kg Sch B treated group had higher frequencies to enter the open arm (Fig. 1B). The mice with Sch B treatment exhibited a higher percentage of time in the central area as compared with the model group (Fig. 1D). As for Sch B treated group, the total arm entries in the EPM and the total distance in the OFT have no change in contrast to the model group (Fig. 1C, 1E).

3. Effect of Sch B on MDA and ROS levels of stress-induced anxiety mice.
The oxidative stress markers, MDA and ROS levels of serum and amygdala were assessed by the assay kits. From the results, MDA and ROS levels of serum (Fig. 2A-B) and amygdala (Fig. 2C-D) in model group were significantly increased compared with the control one, and the changes can be rectified by the administration of Sch B, especially higher dose group (60 mg/kg). The Sch B process obviously antioxidant capacity.

4. Effect of Sch B on SOD activity and GSH level of stress-induced anxiety mice.

The antioxidant markers, such as SOD and GSH, play an important role in the central neuron system. We detected SOD activity and GSH level of serum and amygdala in four groups. To our surprise, the SOD activity (Fig. 3A, 3C) and GSH content (Fig. 3B, 3D) of the model group are in a lower level as compared with the control group, no matter serum or amygdala. Yet the administration of Sch B reversed the SOD activity and GSH content, and the statistical analysis at higher dose group (60 mg/kg) showed significant difference than the lower one as compared with the model group. This data further verified our hypothesis that Sch B process obviously antioxidant capacity.

5. Effect of Sch B on neuronal damage of stress-induced anxiety mice.

Oxygen metabolism is involved in the generation of ROS and MDA, which could induce cells death. In order to observe histological damage in the amygdala, the Nissl staining was used to exhibit a visual interpretation of pyknotic cells. The obvious difference of cellular morphology between the different groups of mice was showed in the Fig. 4A. Quantitative analysis of damage within the amygdala revealed a significant difference in the proportion of intact neurons in model mice as compared to control group. Both 30 mg/kg and 60 mg/kg Sch B treatment groups reduced the amount of damage in the amygdala (Fig. 4B).
6. **Effect of Sch B on the Nrf2/Keap1 expression in stress-induced anxiety mice.**

The Nrf2 pathway has been identified to be the pivotal regulator for oxidative stress, which participates in transcription of many enzymes, such as SOD and GPX (Vnukov et al. 2017). To explore the exact mechanism of Sch B neuroprotective effect in the anxiety disorder, the western blot method was used to verify the conjecture that whether Sch B altered expression of molecular targets commonly associated with oxidative stress. We quantified the expressions of Nrf2 and Keap1, a negative regulatory protein inhibiting the activity of Nrf2. Immunoblot results showed that both 30 mg/kg and 60 mg/kg Sch B improved Nrf2 expression that was decreased in the model group (Fig. 5A). The gray scan analysis further confirmed the changes of Nrf2 expression among four groups (Fig. 5B). Meanwhile, up-regulation of Keap1 protein can be reversed by Sch B, especially the high-dose group (Fig. 5C).

**Discussion**

Anxiety-related disorders are complex illnesses that underlying molecular mechanisms need to be understood. The amygdala stand as an important link between emotional input and output, and its dysfunction is obviously associated to anxiety (Davidson 2002, Davis et al. 2010). Previous research reported that the forced swimming, an acute stressor, causes cell loss in some brain regions such as amygdala (Eggers 2007, Sajja et al. 2015) and further induced anxiety-like behaviors (Tian et al. 2013). The present behavioral data provided some evidence for the force swimming induced anxiety. In addition, redox reactions are a crucial component of many natural physiological processes, however, sustained oxidative stress is associated with a number of diseases including Parkinson's and Alzheimer's diseases (Li et al. 2017).
235 Our results showed that the level of oxidative stress markers (MDA & ROS) was increased compared with model group, whether in serum or the amygdala. Consistent with the general conclusion (Kumar et al. 2014, Shahzad et al. 2014), the oxidative stress damage is also an important pathogenesis of anxiety.

239 Sch B, as a traditional Chinese herb, has been shown to afford generalized tissue protection against oxidative damage in various organs (Lam and Ko 2012, Giridharan et al. 2015). We found the administration of Sch B reversed abnormal behaviors in force swimming group (Fig 1). Meanwhile, the antioxidant system, SOD activity and GSH level were increased and oxidative substances markers decreased (Fig 3), along with the less pyknotic cell morphology and neuron cell death in the Sch B treatment groups through Nissl staining (Fig 4), portends that Sch B have a protective effect for the neuron of amygdala. Furthermore, we speculated that anxiolytic effect of Sch B may be associated with the neuroprotective effect of Sch B in the amygdala. There is a close relationship between oxidative stress and the development of affective disorders, such as anxiety in our present research.

249 The antioxidation system, such as SOD and GSH, can be regulated by Nrf2/Keap1 cascade response signaling pathway (Ma 2013). The nuclear factor Nrf2 not only limits to antioxidative transactivation, but also plays an important role in encountering various kinds of physiological and pathological stress (Chiu et al. 2011, Leong et al. 2011). Degradation of Nrf2 is triggered through the Keap1/Cul3 ubiquitin ligase (Ma 2013). In our research, we found that Nrf2 level was increased and Keap 1 was decreased after Sch B treatment (Fig 5), is in agreement with the change of SOD activity and GSH level. The Sch B plays an antioxidant role via modulating some signal pathway, especially the Nrf2 pathway.
previous study reported that Sch B reversed SOD activity and GSH level through Nrf2 pathway and exhibited the protective effects on cigarette smoke-induced lung inflammation (Jia et al. 2017). The antioxidative function of Sch B not only in peripheral nervous system, but in the CNS. The administration of Sch B revealed a rescue regulation between oxidation and antioxidation system. The data further verified our conjecture that Sch B therapy has a protective role against oxidative stress probably through intervention of Nrf2/Keap 1 pathway. A significant correlation between Sch B and Nrf2 pathway further highlight the importance of the Nrf2/Keap 1 pathway.

In conclusion, Sch B increased Nrf2 expression and decreased Keap1 protein levels, closely linked with mental state and anxiety. Furthermore, Sch B administration rescued the abnormal level of oxidative products and visible histological damage in the amygdala, and anxiety-like symptoms induced by the force swimming has been relieved. However, the exact mechanism that how Sch B triggered Nrf2 antioxidant signaling pathway and whether the pathway is a direct way to alleviate the anxiety still unclear, future studies will be needed to produce a more thorough understanding of the mechanisms involved and influences in other brain regions.

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Conflict of interest

The authors have no conflict of interest.

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**Figure captions**

**Fig. 1** Sch B relieves anxiety-like behavior caused by the acute stress. (A-C) The behaviors of four group in the EPM; (D-E) The behaviors of four group in the OFT. n=10, results are presented as mean ± S.E.M. *p < 0.05, **p < 0.01 as compared to control group; #p < 0.05, ##p < 0.01 as compared to model group.

**Fig. 2** Sch B reversed MDA and ROS levels that were increased in the stress-induced anxiety mice. (A) Serum MDA content (nmol/ml); (B) Serum ROS level (% of control). (C) MDA in the amygdala (pmol/mg); (D) ROS level in the amygdala (% of control). n=10, results are presented as mean ± S.E.M. *p < 0.05, **p < 0.01 as compared to control group; #p < 0.05, ##p < 0.01 as compared to model group.

**Fig. 3** Sch B increased SOD activity and GSH levels in the stress-induced anxiety mice. (A) Serum SOD activity (U/ml); (B) Serum GSH level (nmol/ml). (C) SOD activity in the amygdala (U/mg); (D) GSH level in the amygdala (umol/mg). n=10, results are presented as mean ± S.E.M. *p < 0.05, **p < 0.01 as compared to control group; #p < 0.05, ##p < 0.01 as compared to model group.

**Fig. 4** Morphological evaluation in the amygdala by Nissl staining. (A) The amygdala region of control group (at 10×). The cellular morphology of amygdala in different groups is showed by using a stereomicroscope (at 60×) (B) Quantitative analysis of the proportion of intact neurons. Ten different regions (including approximately 30-40 cells each) were counted in every photograph of amygdala. Results are presented as mean ± S.E.M. *p < 0.05, **p < 0.01 as compared to control group; #p < 0.05, ##p < 0.01 as compared to model group. by using a

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389  stereomicroscope

390  **Fig. 5** Effect of Sch B on Nrf2 and Keap1 protein expression. (A) Band images of Nrf2 and Keap1; (B) Intensity analysis of Nrf2; (C) Intensity analysis of Keap1. n=10, results are presented as mean ± S.E.M. *p < 0.05, **p < 0.01 as compared to control group; #p < 0.05, ###p < 0.01 as compared to model group.
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