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Effect of Suan Zao Ren (Semen Ziziphi Spinosae) Extract on the TXNIP/NLRP3 Pathway in Insomniac Rats

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Abstract

Objectives This study aimed to investigate the therapeutic effects of Suan Zao Ren (Semen Ziziphi Spinosae, SZS) extract on insomnia induced by *p*-chlorophenylalanine (PCPA) in rats and its influence on the thioredoxin-interacting protein (TXNIP)/nucleotide-binding domain Leucine-rich repeat and pyrin domain-containing receptor 3 (NLRP3) inflammasome pathway, and to preliminarily explore the mechanism by which SZS extract improves insomnia.

Methods Fifty male Sprague–Dawley (SD) rats were used, with 8 rats in the blank group and 42 rats in the modeling group. The modeling group was induced by intraperitoneal injection of PCPA at a dose of 500 $mg \cdot kg^{-1}$ for six consecutive days, with daily cage exchange. After 6 days, 40 successfully modeled rats were randomly divided into five groups: the model group (equal volume of distilled water), the positive group (0.75 $mg \cdot kg^{-1}$), and low-, medium-, and high-dose SZS extract groups (1.5, 3, and 6 $g \cdot kg^{-1}$, respectively), with 8 rats in each group. Treatments were administered for seven consecutive days. Enzyme-linked immunosorbent assay was used to measure levels of 5-hydroxytryptamine (5-HT) and gamma-aminobutyric acid (GABA) in the rat cerebral cortex. The thiobarbituric acid (TBA) method was used to determine malondialdehyde (MDA) levels, and the hydroxylamine method was used to determine superoxide dismutase (SOD) levels. The 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) method was used to measure total antioxidant capacity (TAOC) in the cerebral cortex. Pathological changes in the cerebral cortex were observed, and Western blot was used to detect the protein expressions of TXNIP, NLRP3, apoptosis-associated speck-like protein containing a Caspase activation and recruitment domain (CARD), and cysteine–aspartate-specific protease 1 (Caspase-1) in the cerebral cortex.

Results Compared with the blank group, the model group showed a significantly prolonged sleep latency ($p < 0.001$) and a significantly shortened sleep duration ($p < 0.001$). There were no changes in serum MDA and SOD levels. MDA levels in the cerebral cortex were significantly increased ($p < 0.001$), while SOD and TAOC levels were significantly decreased ($p < 0.001$). The 5-HT level was increased ($p < 0.05$), and the GABA level was significantly decreased ($p < 0.001$). SZS extract improved these conditions to varying degrees. Light microscopy showed no significant changes in

Keywords
- insomnia
- Suan Zao Ren
- TXNIP/NLRP3
- oxidative stress
- sedative and hypnotic

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cortical neurons but transmission electron microscopy revealed intact mitochondrial structures in the blank group, while the model group showed swollen and unclear mitochondria with reduced organelles. After 7 days of treatment, these conditions improved in the SZS extract groups. Compared with the blank group, the expressions of the four proteins in the model group were increased, and the expressions of these proteins were decreased in the SZS extract groups compared with the model group.
Conclusion SZS extract may exert an antioxidant effect to treat insomnia by down-regulating the expression of TXNIP/NLRP3 proteins and regulating oxidative stress levels in the cerebral cortex.

Introduction

Sleep is a basic physiological need for humans, and insomnia is the most common sleep disorder. The global incidence of insomnia is gradually increasing, with reports indicating that 33% to 50% of adults worldwide suffer from insomnia.[1] This has become a global public health issue. The primary medications for treating insomnia are benzodiazepines and non-benzodiazepine drugs.[2,3] However, their clinical efficacy is only 65% to 70%.[4] Long-term use can lead to adverse effects such as addiction, tolerance, hepatotoxicity, withdrawal symptoms, and rebound insomnia upon discontinuation.[5–9] Chinese herbs also show good clinical efficacy in treating insomnia, with Suan Zao Ren being the foremost sedative herb.[10]

Suan Zao Ren (Semen Ziziphi Spinosae, SZS), the dried ripe seed of the Rhamnaceae plant *Ziziphus jujuba Mill. var. spinosa* (Bunge) Hu ex H.F. Chou, has been used since the Eastern Han Dynasty. SZS has the functions of nourishing the heart and liver, calming the heart and mind, suppressing sweating, and generating fluids. It is commonly used for the treatment of diseases such as insomnia, palpitations and dreams, excessive sweating due to physical weakness, and thirst caused by fluid damage. SZS has a sweet and mild taste and can be used alone or in combination for various types of insomnia, with outstanding therapeutic effects. At the same time, studies have found that jujube seeds also have antioxidant effects. In addition, SZS is also one of the medicinal and edible dual-use products. They have a good taste, no obvious toxic side effects, and do not have the addictive, tolerant, or hepatotoxic properties commonly found in sedative and hypnotic drugs. Therefore, they are widely welcomed by doctors and patients. The usage of clinical decoctions has been increasing year by year, and the market is in short supply. As a result, the price of SZS has been increasing year by year. In-depth research on the optimal dosage, active ingredients, mechanism of action, and appropriate dosage form of SZS for insomnia is beneficial for improving the cost-effectiveness of the clinical use of SZS.

Multiple studies have shown an interaction between insomnia and oxidative stress (OS).[11,12] In the brain, OS can cause damage by reactive oxygen species (ROS) destroying various biological molecules like DNA, RNA, lipids, and proteins.[13] OS can induce insomnia, and insomnia can decrease the body's antioxidant capacity, causing damage to various physiological functions and triggering biological effects such as OS and inflammation.

This study establishes a *p*-chlorophenylalanine (PCPA)-induced insomnia rat model to observe the effects of different doses of SZS extract on sleep, 5-hydroxytryptamine (5-HT), gamma-aminobutyric acid (GABA), and OS in insomniac rats. It aims to explore whether the SZS extract affects insomnia through the thioredoxin-interacting protein (TXNIP)/nucleotide-binding domian Leucine-rich repeat and pyrin domian-containing receptor protein 3 (NLRP3) signaling pathway, thereby preliminarily investigating the mechanism by which SZS extract improves insomnia.

Materials

Animals

Fifty 8-week-old, healthy, male, specific pathogen free (SPF)-grade Sprague–Dawley (SD) rats, weighing (200 ± 20) g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. [License No.: SCXK (Beijing) 2021-0011]. The animals were housed at the Experimental Animal Center of Beijing University of Chinese Medicine in a temperature-controlled environment (22~24 °C) with a humidity of 45% to 55% [License No.: SYXK (Beijing) 2020-0033]. The experiment was reviewed and approved by the Ethics Committee of Beijing University of Chinese Medicine (Approval No.: BUCM-2023112809-4144) and humane care was given following the reduction, replacement and refinement (3R) principles of animal use.

Drugs and Reagents

SZS [Zhenxing Baicao (Beijing, China) Pharmaceutical Co., Ltd. Produced in Hebei Province]; PCPA (Shanghai Rhawn Chemical Technology Co., Ltd., Item No. 7424-00-21, China); pentobarbital sodium (Beijing Chemical Reagent Company, Item No. 060222, China); 5-HT enzyme-linked immunosorbent assay (ELISA) kit, GABA ELISA kit (Jiangsu Kete Biotechnology Co., Ltd., Item No. KT3318-A, KT3317-A, China); radio immuno precipitation assay lysis buffer (RIPA) lysis buffer, BCA protein concentration assay kit, phenylmethylsulfonyl fluoride (PMSF) protease inhibitor, enhanced chemiluminescence (ECL) plus ultrasensitive luminescence solution, superoxide dismutase (SOD) assay kit, malondialdehyde (MDA)

assay kit (Nanjing Jiancheng Bioengineering Institute, Item No. R0020, PC0020, A8260, PE0010, A001-1, A003-1, China); total antioxidant capacity (TAOC) assay kit (Jiangsu Edison Biotechnology Co., Ltd., Item No. ADS-W-YHKY004-96, China); eosin staining solution, hematoxylin staining solution, 2.5% electron microscopy tissue fixative (Beijing Solarbio Co., Ltd., Item No. G1100, G114, P1116, China); TXNIP (the dilution ratio is 1:1,000), NLRP3 (the dilution ratio is 1:1,000), cysteine–aspartate-specific protease 1 (Caspase-1; the dilution ratio is 1:1,000), apoptosis-associated speck-like protein containing a Caspase activation and recruitment domain (CARD) (ASC; the dilution ratio is 1:1,000), HRP-labeled anti-mouse secondary antibody (the dilution ratio is 1:16,000), HRP-labeled anti-rabbit secondary antibody (the dilution ratio is 1:10,000) (Wuhan Sanying Biotechnology Co., Ltd., Item No. ptg18243-1-AP, ptg27458-1-AP, ptg22915-1-AP, ptg67494-1-1g, RGAM001, RGAR001, China).

Instruments and Equipments

Optical microscope (DM1000, Leica Microsystems, Ltd., Germany); Benchtop refrigerated centrifuge (D06847, Beckman, United States); Paraffin microtome (RM2135, Leica Microsystems, Ltd., Germany); Transmission electron microscope (H-7650, Hitachi, Japan); Synergy II microplate reader (800TS, BioTek, United States); Bio-Rad vertical electrophoresis system and Bio-Rad wet transfer system (Bio-Rad, United States) were used in the study.

Methods

Drug Preparation

SZS extract was prepared using 50% ethanol extraction. To establish the model, a suspension of 500 $mg \cdot kg^{-1}$ weakly alkaline PCPA was prepared, with Arabic gum added to aid dissolution. The suspension was agitated in a constant temperature shaker for 48 hours.

Model Establishment, Verification, and Grouping

Out of the 50 rats, 42 were designated as the model rats and were intraperitoneally injected with PCPA (10 mL/kg). The cages were swapped daily to provide mild stimulation, once a day for six consecutive days. The remaining eight rats served as the blank group (N group) and received no treatment. The success of the model was verified by observing the general state of the rats and conducting a pentobarbital sodium-assisted sleep experiment. Compared with the N group, the general condition of the modeling rats deteriorated significantly, with dry fur and easy shedding, increased irritability, heightened vigilance, and aggression (see ►**Table 1**). In addition, the sleep latency of the model rats was significantly prolonged ($p < 0.001$), and the sleep duration was significantly shortened ($p < 0.001$) compared to the N group (see ►**Table 2**). These comprehensive results indicate that the insomnia model has been successfully established.

After excluding two rats that failed to model successfully, the remaining 40 rats were randomly divided into the following groups: model group (M group), Positive control group (P group), high-dose SZS extract group (SH group), medium-dose SZS extract group (SM group), and low-dose SZS extract group (SL group). The P group was given 0.75 $mg \cdot kg^{-1}$ of zopiclone, while the SH, SM, and SL groups were given 6, 3, and 1.5 $g \cdot kg^{-1}$ of Suan Zao Ren (SZS), respectively. Each administration group received their respective drugs via gavage at 5 mL/kg body weight, while the N and M groups received the same volume of distilled water by gavage. The administration was done once daily for 7 consecutive days, with the general state of the rats being closely observed during this period.

Pentobarbital Sodium-assisted Sleep Experiment

Sixteen hours after the last administration, a pentobarbital sodium-assisted sleep experiment was conducted to observe the sleep conditions of the rats in each group. All rats were intraperitoneally injected with 0.8% pentobarbital sodium (40 $mg \cdot kg^{-1}$). The time from drug administration to the disappearance of the righting reflex and the natural drooping of the tail was recorded as the sleep latency period. The period from sleep onset to the recovery of the righting reflex was recorded as the sleep duration period. Sleep latency and duration times were recorded for each group.

Table 1 General state comparison of rats after model making

Group	*n*	Status	Activity amount	Fur condition	Irritability	Aggressive behavior
N group	8	Good condition	Normal	Shiny fur	Not easy to be provoked	No
Modelling group	40	Poor state	Hyperactivity	Dry fur, easily shed	Easy to be provoked	Yes

Abbreviation: N group, blank group.

Table 2 Sleep latency and sleep duration in rats after model making ($\overline{x} \pm s$, min)

Group	*n*	Sleep latency	Sleep duration
N group	8	2.79 ± 0.39	238.15 ± 28.37
Modelling group	40	4.69 ± 1.21[a]	134.29 ± 35.84[a]

Abbreviation: N group, blank group.
[a]$p < 0.001$, compared with N group.

Sample Collection

Samples were collected after 7 days of administration. The rats were fasted for 12 hours before sampling but allowed water. Two rats from each group were perfused, and their brains were immediately immersed in fixative for 24 hours for histological observation. The remaining rats were anesthetized with 1% pentobarbital sodium (40 mg·kg^{-1}), and blood was collected from the abdominal aorta. The blood was left to stand for 4 h, then centrifuged at 3,000 r·min^{-1} for 15 min, and the serum was stored at −80 °C for later testing. After blood collection, the rats were quickly decapitated, and the entire brains were removed and placed on ice. The cerebral cortex was separated and stored in freezing tubes at −80 °C for later use.

Determination of Malondialdehyde and Superoxide Dismutase Levels in Serum

Serum at a concentration of 10% was used to determine MDA levels. Serum at a concentration of 20% was used to determine the SOD levels. MDA levels were determined using the TBA method, and SOD levels were determined using the hydroxylamine method. The procedures were strictly followed according to the kit instructions.

Determination of Malondialdehyde, Superoxide Dismutase, and Total Antioxidant Capacity Levels in the Cerebral Cortex

The cortical tissue was made into 10% tissue homogenate, and the supernatant was removed. The MDA and SOD detection methods were the same as above. TAOC levels were measured using the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay. ABTS assay is commonly used for antioxidant activity. ABTS is oxidized to green $ABTS^+$ under the appropriate oxidant, while $ABTS^+$ production will be inhibited in the presence of antioxidants, and the absorbance of $ABTS^+$ at 734 or 414 nm can be measured and calculated the TAOC of the sample.

Determination of 5-Hydroxytryptamine and gamma-Aminobutyric Acid Levels in the Cerebral Cortex

The cortical tissue was homogenized into 10% tissue homogenate, and the supernatant was removed for the determination of 5-HT and GABA levels. The levels of 5-HT and GABA in each group were measured by a double-antibody sandwich using a kit.

Histological Observation of the Cerebral Cortex

The cerebral cortex was fixed in 10% formaldehyde, dehydrated in an ethanol gradient, transparent in xylene, embedded in paraffin, and then made into 3μm paraffin sections, which were stained with HE staining and observed by a light microscope. The cerebral cortex tissue less than 1 mm^3 was fixed in 2.5% glutaraldehyde for 1 to 2 h, then washed twice with 0.1 mol·L^{-1} Phosphate Buffered Saline (PBS) at 4 °C, fixed in 1% osmium acid for 1 to 2 h, and washed twice with 0.1 mol·L^{-1} PBS, and then dehydrated with 50%, 70%, and 90% ethanol for 5 to 10 min each time, and then dehydrated in 100% ethanol for three times at room temperature, and then soaked with 100% ethanol:propylene oxide = 1:1 for 10 min at room temperature, soaked with propylene oxide for 10 min at room temperature, soaked with Epon 812:propylene oxide = 1:1, and then embedded in Epon 812 after more than 2 h in vacuum desiccator (0.2–0.4 kg·cm^{-2}). The tissues were polymerized at 40 °C for 2 h, 60 °C for 4 h, and 80 °C for 10 h. Ultrathin sections were stained with uranium and lead for 15 min and 10 min, respectively, and observed under a transmission electron microscope.

Determination of TXNIP, NLRP3, ASC, and Caspase-1 Protein Expression in the Cerebral Cortex

The cerebral cortex tissue was homogenized in RIPA lysis buffer, transferred to eppendorf (EP) tubes, and lysed on ice for 30 min, centrifuged at a speed of 12,000 r·min^{-1} at 4 °C with a radius of 9.5 cm, and then the supernatant was collected, and sample buffer was added. The samples were mixed by using a high-speed vortex and denatured at 95 °C for 5 min. SDS-PAGE was used for electrophoresis and wet transfer. The membranes were blocked with 5% skim milk, incubated with primary antibodies overnight at 4 °C, and secondary antibodies at room temperature for 1 h. The bands were visualized, and the gray values were analyzed to determine protein expression.

Statistical Methods

Data were processed using SPSS 23.0 software. Measurement data conforming to a normal distribution were expressed as mean ± standard deviation ($\bar{x} \pm s$). One-factor analysis of variance (*ANOVA*) was used for comparisons among multiple groups, and least–significant difference (*LSD*) analysis was used for pairwise comparisons.

Results

Effects of Semen Ziziphi Spinosae Extract on Sleep in Rats

As shown in ►**Fig. 1**, compared to the N group, the sleep latency of the M group was significantly prolonged ($p < 0.001$), and the sleep duration was significantly shortened ($p < 0.001$). After 7 days of administration, compared to the M group, the sleep latency of rats in each treatment group was significantly shortened ($p < 0.001$), and the sleep duration was significantly prolonged ($p < 0.001$). There were some slight but not statistically significant differences between the SZS dose group.

Effects of Semen Ziziphi Spinosae Extract on Serum Malondialdehyde and Superoxide Dismutase Levels in Rats

As shown in ►**Fig. 2**, there were no significant differences in MDA and SOD levels between the M group and the N group. Therefore, the serum levels of MDA and SOD in the treated rats were not tested later.

Effects of Semen Ziziphi Spinosae Extract on Malondialdehyde, Superoxide Dismutase, and Total Antioxidant Capacity Levels in the Cerebral Cortex of Rats

As shown in ►**Fig. 3**, compared to the N group, the MDA level in the M group was significantly increased ($p < 0.001$), and

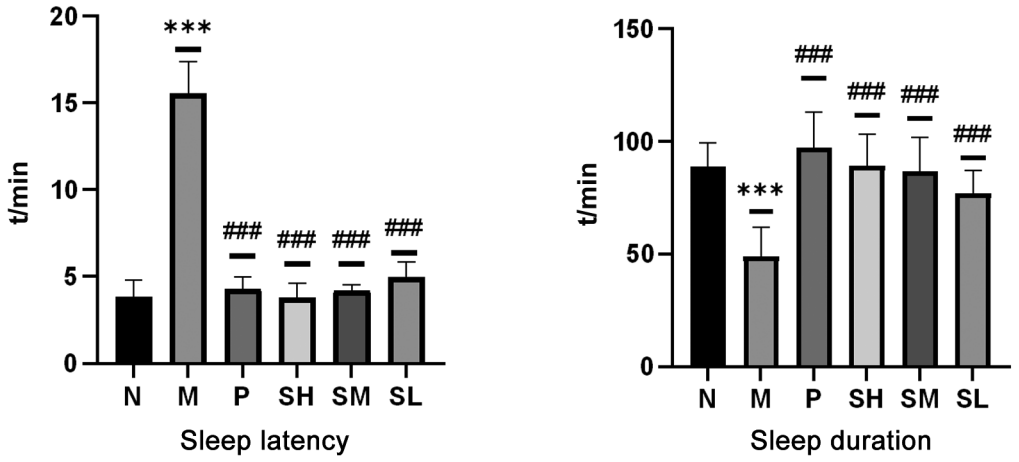

Fig. 1 Effect of SZS extract on sleep condition. Compared with N group, $^{***}p < 0.001$; compared with M group, $^{###}p < 0.001$. M, model group; N, blank group; P, positive control group; SH, high-dose SZS extract group; SL, low-dose SZS extract group; SM, medium-dose SZS extract group; SZS, Semen Ziziphi Spinosae.

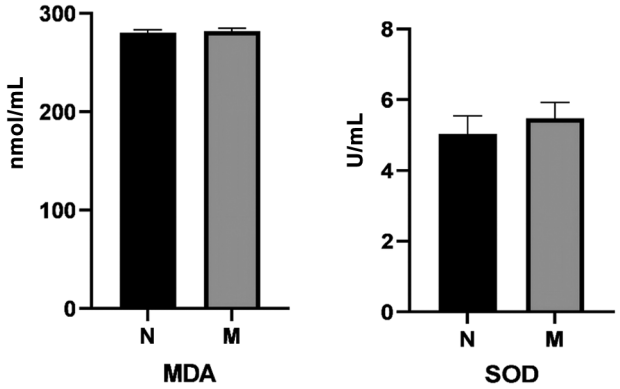

Fig. 2 Effect of SZS extract on serum MDA and SOD. M, model group; MDA, malondialdehyde; N, blank group; SOD, superoxide dismutase; SZS, Semen Ziziphi Spinosae.

the levels of SOD and TAOC were significantly decreased ($p < 0.001$). After administration, compared to the M group, the levels of MDA in the cerebral cortex of the SH and SM groups rats were significantly reduced ($p < 0.001$), the SOD concentration in the SH group rats was significantly increased ($p < 0.001$), and the TAOC level was also increased in the SH group ($p < 0.05$). However, there were no significant effects on SOD and TAOC levels in the SM and SL groups rats.

Effects of Semen Ziziphi Spinosae Extract on 5-Hydroxytryptamine and gamma-Aminobutyric Acid Levels in the Cerebral Cortex of Rats

As shown in ►**Fig. 4**, compared to the N group, the 5-HT level in the M group was higher ($p < 0.05$), and the GABA level was significantly lower ($p < 0.001$). Compared to the M group, the SH group significantly improved the 5-HT and GABA levels ($p < 0.05$). There were no significant differences between the SH and SM groups in their effects on these neurotransmitters.

Effects of Semen Ziziphi Spinosae Extract on the Structure of the Cerebral Cortex in Rats

In the HE staining (see ►**Fig. 5**), the cerebral cortex structure of rats in the N group was clear and intact, while there were no significant changes observed in the other groups. Transmission electron microscopy (see ►**Fig. 6**) revealed that the

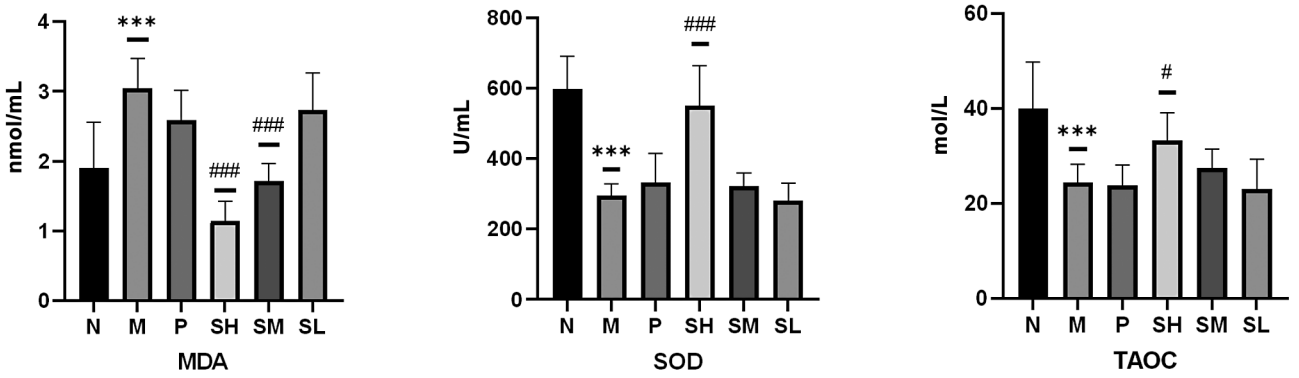

Fig. 3 Effect of SZS extract on MDA, SOD, and TAOC. Compared with N group, $^{***}p < 0.001$; compared with M group, $^{\#}p < 0.05$, $^{###}p < 0.001$. M, model group; MDA, malondialdehyde; N, blank group; P, positive control group; SH, high-dose SZS extract group; SL, low-dose SZS extract group; SM, medium-dose SZS extract group; SOD, superoxide dismutase; SZS, Semen Ziziphi Spinosae; TAOC, total antioxidant capacity.

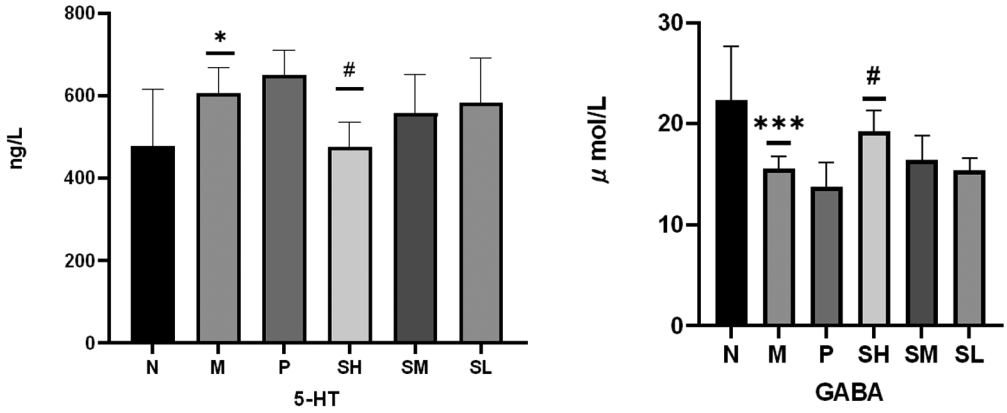

Fig. 4 Effect of SZS extract on 5-HT and GABA. Compared with N group, $^{*}p < 0.05$, $^{***}p < 0.001$; compared with M group, $^{\#}p < 0.05$. 5-HT, 5-hydroxytryptamine; GABA, gamma-aminobutyric acid; M, model group; N, blank group; P, positive control group; SH, high-dose SZS extract group; SL, low-dose SZS extract group; SM, medium-dose SZS extract group; SZS, Semen Ziziphi Spinosae.

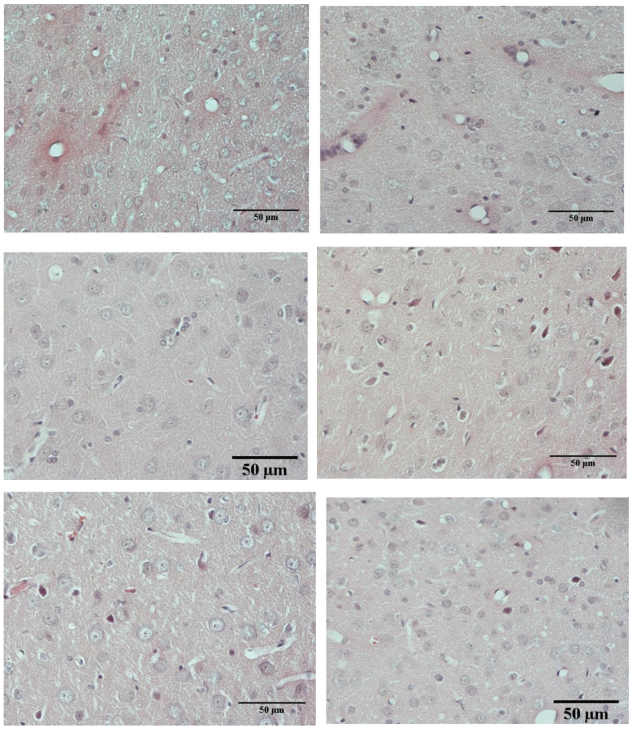

Fig. 5 Structure of neural cells in the cerebral cortex (HE, ×40, scale = 50 μm). Pictures are successively shown as N, M, P, SH, SM, and SL. M, model group; N, blank group; P, positive control group; SH, high-dose SZS extract group; SL, low-dose SZS extract group; SM, medium-dose SZS extract group; SZS, Semen Ziziphi Spinosae.

mitochondrial structure was intact in the cortex of the N group. In contrast, the mitochondria in the M group were swollen and blurred, with most organelles even disappearing. After 7 days of administration, the degree of mitochondrial swelling was significantly improved compared to the M group.

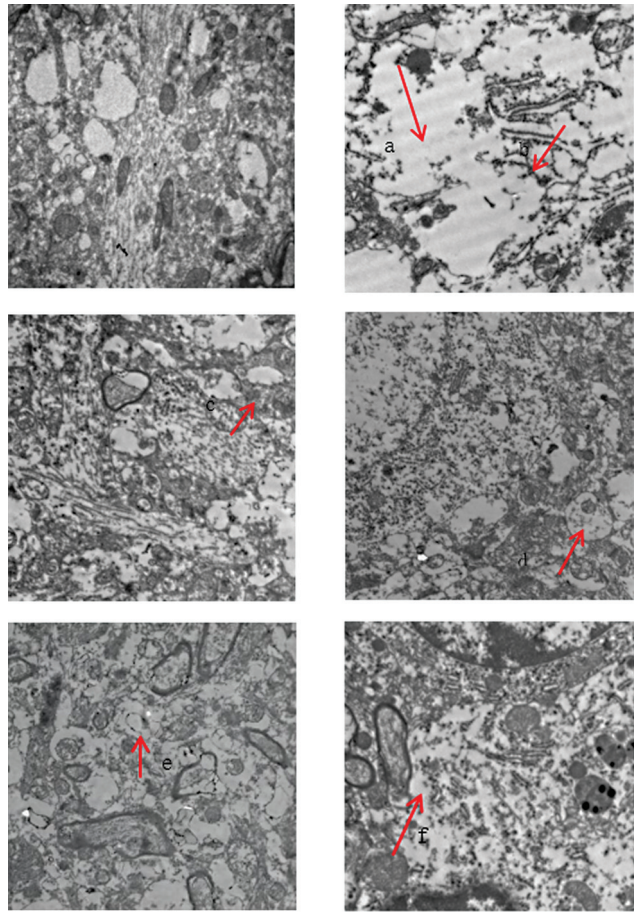

Fig. 6 The cerebral cortex neural cells of each group were structured under electron microscopy (×15,000). Pictures are successively shown as N, M, P, SH, SM, and SL; (a) disappearance of organelles; (b) rupture of mitochondrial cristae; (c) mitochondrial vacuolation; (d) mitochondrial swelling; (e) rupture of mitochondrial membrane; and (f) dissolution of organelles. M, model group; N, blank group; P, positive control group; SH, high-dose SZS extract group; SL, low-dose SZS extract group; SM, medium-dose SZS extract group; SZS, Semen Ziziphi Spinosae.

Effects of Semen Ziziphi Spinosae Extract on TXNIP, NLRP3, ASC, and Caspase-1 Protein Expression in the Cerebral Cortex of Insomniac Rats

►**Table 3** and ►**Fig. 7** shows that, compared with N group, the expression levels of TXNIP, NLRP3, ASC, and Caspase-1 proteins in the M group were significantly increased ($p < 0.001$). Compared with the M group, the protein expression levels of TXNIP, NLRP3, ASC, and Caspase-1 were significantly decreased in the SH and SM groups ($p < 0.05$).

Table 3 Fig. 7 Effects of Semen Ziziphi Spinosae extract on TXNIP, NLRP3, ASC, and Caspase-1 protein ($\bar{x} \pm s$, $n = 3$)

Group	TXNIP/GAPDH	NLRP3/GAPDH	ASC/GAPDH	Caspase-1/GAPDH
N	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
M	1.95 ± 0.30^{a}	2.01 ± 0.27^{a}	2.18 ± 0.2^{a}	2.39 ± 0.26^{a}
P	1.79 ± 0.25	1.63 ± 0.93	1.39 ± 0.48^{d}	1.38 ± 1.01^{c}
SH	1.11 ± 0.21^{d}	1.10 ± 0.18^{b}	1.13 ± 0.07^{d}	1.11 ± 0.45^{d}
SM	1.23 ± 0.10^{d}	1.22 ± 0.09^{b}	1.31 ± 0.14^{d}	1.73 ± 0.18^{b}
SL	1.83 ± 0.19	1.38 ± 0.35	1.85 ± 0.14	2.16 ± 0.15

Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; Caspase-1, cysteine–aspartate-specific protease 1; M, model group; N, blank group; NLRP3, nucleotide-binding domian Leucine-rich repeat and pyrin domain-containing receptor 3; P, positive control group; SH, high-dose SZS extract group; SL, low-dose SZS extract group; SM, medium-dose SZS extract group; SZS, Semen Ziziphi Spinosae; TXNIP, thioredoxin-interacting protein.
Compared with N group: $^{a}p < 0.001$.
Compared with M group: $^{b}p < 0.05$; $^{c}p < 0.01$; $^{d}p < 0.001$.

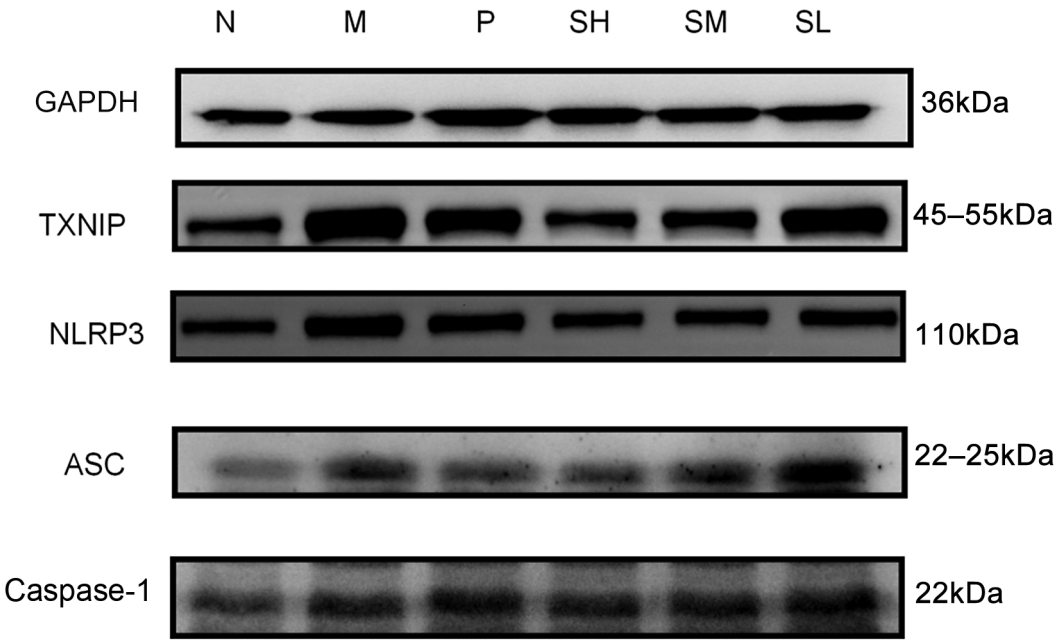

Fig. 7 Protein expression of TXNIP, NLRP 3, ASC, and Caspase-1 in the cerebral cortex. ASC, apoptosis-associated speck-like protein containing a CARD; Caspase-1, cysteine–aspartate-specific protease 1; M, model group; N, blank group; NLRP3, nucleotide-binding domian Leucine-rich repeat and pyrin domain-containing receptor protein 3; P, positive control group; SH, high-dose SZS extract group; SL, low-dose SZS extract group; SM, medium-dose SZS extract group; SZS, Semen Ziziphi Spinosae; TXNIP, thioredoxin-interacting protein; $n = 3$.

However, there was no change in the expression levels of each protein in the SL group.

Discussion

The PCPA insomnia rat model is currently recognized in the academic community as a valid animal model for insomnia. PCPA, as a tryptophan hydroxylase inhibitor, can inhibit the synthesis of the neurotransmitter 5-HT in the rat brain, causing the rats' circadian rhythm to disappear.[14–16] Typically, the modeling duration is about 3 days but the success rate is low. Additionally, due to the low solubility of PCPA in water, it tends to clog the needle during injection when prepared as a suspension. Therefore, we modified the modeling method. Based on the literature review, we prepared a weak alkaline saline solution using sodium bicarbonate tablets, added PCPA powder, stirred it, and then added 10% Arabic gum as a solubilizer, maintaining it in a 4 °C constant temperature shaker at 100 r·min^{-1} for 24 h.[17] To better simulate human insomnia, we combined PCPA intraperitoneal injection with the cage-switching method to simulate the impact of environmental changes on sleep, which is more consistent with real-life stress situations.

After intraperitoneal injection of PCPA, the rats showed poor state, increased activity, dry and easily shedding hair, heightened sensitivity to external stimuli such as sound and light, increased aggression, and irritability. These symptoms were accompanied by significantly prolonged sleep latency and significantly shortened sleep duration, indicating that

the rats developed significant sleep disorders suitable for related studies.

Sleep is a basic physiological need for humans. Adequate sleep can eliminate fatigue, restore energy, and improve immune function. During sleep, tissue and organ self-repair accelerates, which can remove the invading pathogens from the body. Enhanced antioxidant capacity during sleep can delay aging, and the release of growth hormones promotes growth and development in children. However, increasing pressure has made insomnia a global issue. Long-term insomnia can cause various physical and mental damages, such as decreased immunity, irritability, fatigue, headaches, memory decline, anxiety, and depression, significantly affecting the quality of life and work efficiency of insomniacs. Currently, insomnia treatment includes pharmacological and non-pharmacological therapies. Non-pharmacological therapies, such as cognitive–behavioral therapy, acupuncture, massage, and qi-gong therapy are inconvenient to operate and have limited efficacy. Therefore, pharmacological therapy remains the primary treatment for insomnia, supplemented by non-pharmacological therapies. Western medications are fast-acting but come with significant side effects and their overall efficacy is less than satisfactory after prolonged use. Traditional Chinese medicine, known for its safety, effectiveness, and fewer side effects, is favored by patients.

SZS has been used since the Eastern Han Dynasty. It is neutral, tastes sweet and a little sour, and is known to calm the heart, tranquilize the mind, and nourish the liver blood according to the traditional theory. Modern research shows that SZS contains various medicinal components, such as saponins, flavonoids, alkaloids, fatty acids, and amino acids. It has hypnotic, anticonvulsant, antidepressant, anxiolytic, immune-enhancing, and antitumor effects, helping patients improve sleep quality and fall asleep faster. It can also effectively alleviate anxiety and treat neurasthenia. However, the clinical dosage of SZS has been increasing over the years, with the maximum dosage reaching up to 200 g.[18] Is this really necessary?

Observing the effects of SZS extract on sleep in insomniac rats, we found that different dosage groups of SZS extract could shorten sleep latency and prolong sleep duration. This indicates that SZS extract is effective in both difficulty falling asleep and maintaining sleep. However, there was no significant difference among the dosage groups, suggesting that the efficacy of SZS for insomnia might not be dose-dependent. The necessity of high clinical doses of SZS needs further investigation. Zopiclone, a non-benzodiazepine sedative–hypnotic drug, serves as a positive control group due to its short-acting nature, weak next-day residual effects, and fewer side effects. Observations showed no significant difference in the efficacy between the various dosage groups of SZS and zopiclone.

Neurotransmitters 5-HT and GABA play important roles in regulating the sleep–wake rhythm and central fatigue. 5-HT can amplify the body's fatigue information, leading to fatigue sensation and sleep intention. After PCPA injection, the increased monoamine oxidase activity in the rat brain accelerates 5-HT degradation, reducing 5-HT levels, keeping the body awake, and making it difficult to fall asleep or stay asleep. Compared to the N group, the 5-HT levels in the M group increased, and the 5-HT levels among the SZS extract groups increased progressively from SH to SL. This result seems to contradict the principle of the PCPA model. We speculate that after PCPA depletes 5-HT, the imbalance of neurotransmitters in the rats might trigger the compensatory secretion of 5-HT, and SZS extract can effectively restore 5-HT balance in the rat brain. GABA can bind to receptors on nerve cell membranes, inhibiting the excitability of nerve cells and promoting sleep. Compared to the N group, GABA levels in the M group significantly decreased, while the SH group could increase GABA levels in the brain, inhibiting cortical nerve excitement and promoting sleep. Both the direct observation from the pentobarbital sodium-assisted sleep experiment and the neurotransmitter detection proved that SZS extract could promote and maintain sleep.

MDA is the final product of lipid peroxidation reactions, and its level can effectively reflect tissue damage. Studies have found that when oxidative damage in the body worsens, MDA levels increase accordingly. SOD can effectively protect vascular endothelial cells from oxygen free radical damage and regulate the body's OS response, considered a relatively stable and easily obtainable OS marker. By detecting serum MDA level and SOD activity, we found no differences, suggesting that insomnia did not significantly affect systemic circulation, mainly affecting the brain. The experiment showed that the MDA level in the cerebral cortex of the M group rats significantly increased, and SOD activity and TAOC levels significantly decreased. This indicates that OS occurred in the brains of insomniac rats. The SH group could significantly reduce MDA levels, and increase SOD activity and TAOC levels in the brain. The SM group also significantly reduced MDA levels but did not significantly affect SOD and TAOC. The SL group did not significantly affect SOD, MDA, and TAOC, indicating that SZS extract has antioxidant effects, with SH and SM groups being more effective.

OS by insomnia produces a large amount of MDA which can cause lipid peroxidation in nerve cells, damage cell structure, and lead to cell function abnormalities. No differences in cortical tissue structure were observed under a light microscope among the groups but under the electron microscope, mitochondrial cristae fractures and vacuole-like degeneration in the cytoplasm were observed in the M group, different dosage groups of SZS extract could improve these conditions to varying degrees, with SH and SM groups being more effective than the SL group.

TXNIP is an alpha-inhibitory protein (alpha-arrestin), it can binds to thioredoxin (Trx), inhibiting its function and leading to an oxidative/antioxidant imbalance.[19] Therefore, abnormal TXNIP levels can adversely affect cellular redox homeostasis, exacerbating the OS process, and making TXNIP a key regulatory protein in the OS pathway. TXNIP is also a key signaling node in inflammation, regulating the activation of the NLRP3 inflammasome.[20]

The NLRP3 inflammasome is a typical representative of the nucleotide-binding and oligomerization domain (NAHCT)-leucine-rich repeat (LRR)-containing proteins (NALPs) family. The NLRP3 inflammasome is a protein

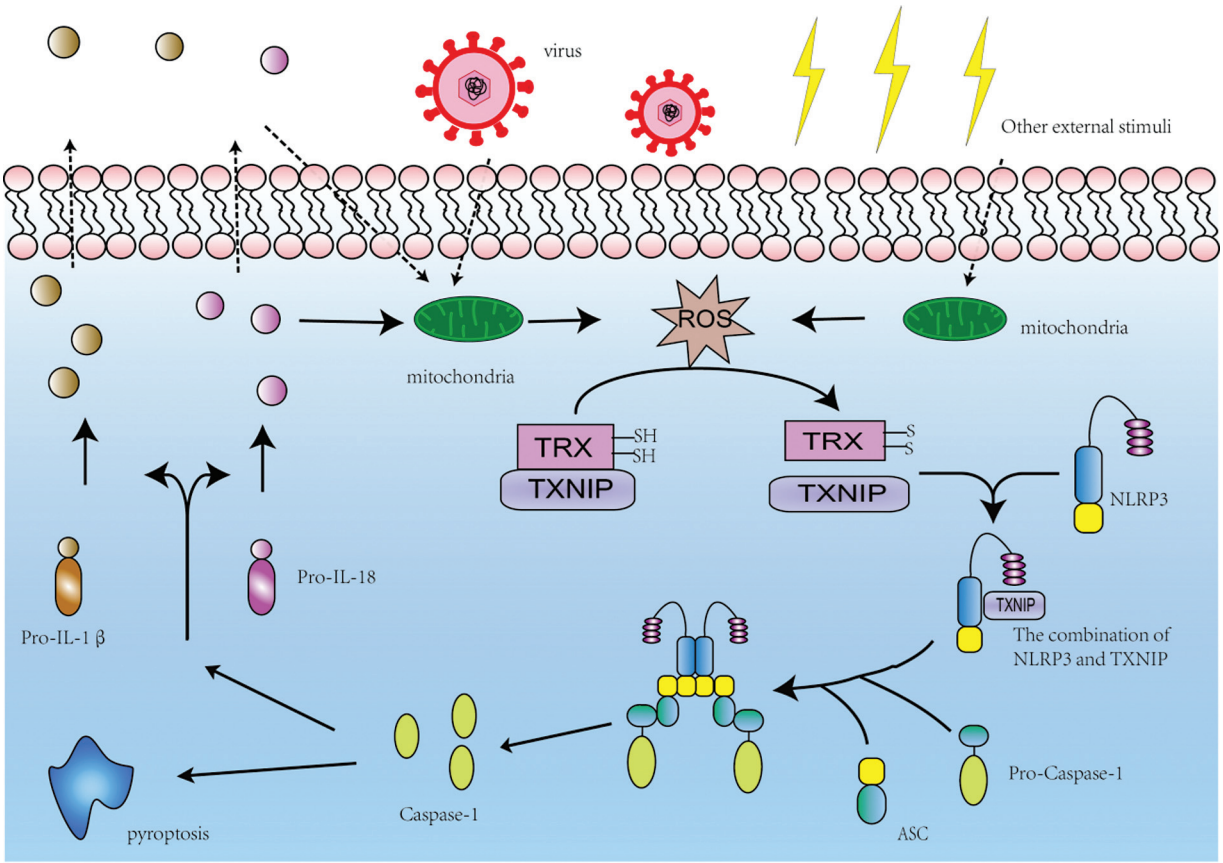

Fig. 8 A Schematic representation of the TXNIP/NLRP 3 signaling pathway. ASC, apoptosis-associated speck-like protein containing a CARD; Caspase-1, cysteine–aspartate-specific protease 1; TRX, thioredoxin; TXNIP/NLRP 3, thioredoxin-interacting protein/nucleotide-binding domian Leucine-rich repeat and pyrin domain-containing receptor 3.

complex composed of NLRP3, ASC, and Caspase-1. When ROS accumulates to toxic levels, oxidized Trx is reduced, causing TXNIP to dissociate and bind to NLRP3. This initiates the assembly of the inflammasome, with the N-terminal Pyrin domain (PYD) of NLRP3 binding to the PYD domain of ASC, forming a homotypic PYD interaction. The CARD domain of ASC binds to the CARD domain of Caspase-1, forming a CARD interaction, with ASC acting as a bridge between the receptor protein and the effector protein. This completes the inflammasome assembly, activating Caspase-1, inducing the maturation of interleukin-1β, enhancing the inflammatory response, and amplifying the OS response caused by insomnia (see ►**Fig. 8**). This study shows that compared to the N group, the expression levels of TXNIP, NLRP3, ASC, and Caspase-1 proteins were elevated in the cortical tissue of the M group. Compared to the M group, the expression levels of these proteins were reduced in the SH and SM groups, suggesting that insomnia activates the TXNIP/NLRP3 signaling pathway, and SZS extract may inhibit the activation of TXNIP/NLRP3 inflammatory pathway by reducing protein expression, thereby protecting cortical neurons.

Conclusion

SZS demonstrated a good sedative effect on insomnia in rats, which could regulate the balance of 5-HT and GABA, reduce the impact of OS, and inhibit the activation of the TXNIP/NLRP3 signaling pathway. This may be a potential mechanism by which SZS extract treats insomnia.

CRediT Authorship Contribution Statement

Zijing Xu: data curation, formal analysis, validation, visualization, and writing—original draft. **Wei Xiong**: formal analysis and visualization. **Qian Wang**: conceptualization, methodology, resources, and writing—review and editing. **Shuyu Li**: resources and conceptualization. **Dexian Jia**: funding acquisition, conceptualization, project administration, supervision, and writing—review and editing.

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Conflict of Interest

The authors declare no conflict of interest.

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