The Effect of *Semen Ziziphi Spinosae* Extract on the p38MAPK/NF-κB Signaling Pathway in Insomniac Rats

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Abstract

Objective The objective of the study was to explore whether Suanzaoren (Semen Ziziphi Spinosae, SZS) extract could improve insomnia by inhibiting the p38 mitogen-activated protein kinase (p38MAPK)/nuclear factor-κB (NF-κB) signaling pathway.

Methods Forty SPF-grade Sprague-Dawley (SD) rats were included in the study, with 10 randomly selected rats serving as the control group. The remaining rats were injected intraperitoneally with p-chlorophenylalanine (PCPA) for 6 days to establish an insomnia model. After successful modeling, the rats were divided into the model group, SZS extract group (3.0 g/kg), and zopiclone group (1.25 g/kg). The rats in the SZS extract and zopiclone groups were administered with the corresponding drugs via gavage for 7 days, while the rats in the control and model groups received distilled water. Sleep latency and sleep duration were recorded, and behavioral changes were observed through elevated plus-maze and open field tests. The levels of oxidative stress markers and serum inflammatory factors were measured by enzyme-linked immunosorbent assay (ELISA). The expression levels of p38 MAPK, p-p38MAPK, p-NF-κBp65, and NF-κBp65 protein in the cerebral cortex were detected by Western blot. Neuronal structures in the cerebral cortex were observed under a transmission electron microscope.

Results Compared with the control group, the model group exhibited abnormal appearances, significant body mass loss ($p < 0.001$), prolonged sleep latency and shortened sleep duration ($p < 0.001$). The SZS extract and zopiclone groups showed significant improvements in these parameters compared with the model group. Compared with the control group, the model group showed significant reduction in total movement distance ($p < 0.001$), fewer entries into the central zone ($p < 0.01$), and significant decrease in rearing frequency ($p < 0.001$); the levels of glutathione peroxidase (GSH-Px) and carnitine acetyltransferase (CT) in the hippocampus were significantly reduced ($p < 0.001$); the serum levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and the expression levels of p-p38MAPK and p-NF-κBp65 in the cerebral cortex were significantly increased ($p < 0.05$). Compared with the model group, the SZS extract group showed significant increase in movement distance ($p < 0.01$) and rearing frequency ($p < 0.001$), significantly increased the GSH-Px and CT levels ($p < 0.001$), and decreased the IL-1β and TNF-α levels ($p < 0.01$); furthermore,

Keywords

- *Semen Ziziphi Spinosae* extract
- p38MAPK/NF-κB signaling pathway
- insomnia
- oxidative stress
- inflammatory factors

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the SZS extract group showed a significantly reduced p-p38MAPK and p-NF-κBp65 levels ($p < 0.05$). The SZS extract group showed significant improvement in the neuronal structure compared with the model group.

Conclusion SZS extract can inhibit the p38MAPK/NF-KB signaling pathway to improve insomnia.

Introduction

Insomnia is the most common sleep disorder in clinical practice, characterized by difficulty in falling asleep and maintaining sleep or experiencing nonrestorative sleep. This condition is often accompanied by daytime symptoms such as fatigue, decreased attention, cognitive impairment, irritability, anxiety, and depression.[1] Recent reports indicated that sleep quality in the Chinese population is generally poor, with half of the population experiencing insomnia and 21% low sleep quality, according to the 2024 China Sleep Health White Paper. Chronic insomnia can severely harm a patient's health and lead to psychological issues such as anxiety and depression, reduced immune function, memory decline, and increased risk of cardiovascular diseases.[2] Insomnia treatment often involves psychological, physical, and pharmacological therapies.[3] However, psychological therapy was slow to take effect, physical therapy lacked large-scale studies, and western medications have significant side effects, limiting their clinical use. Traditional Chinese medicine (TCM), on the other hand, has been shown to be effective in treating insomnia. Semen Ziziphi Spinosae (SZS) is one of the most widely used TCM drugs, known for its ability to nourish the heart, calm the mind, and promote restful sleep.[4] According to drug toxicology studies,[5,6] the SZS extract has no significant toxic reactions and is safe and reliable for clinical use, and suitable for long-term use. This reflects the unique advantages of SZS in treating insomnia.

Studies have shown that insomnia may be related to imbalances in neurotransmitter secretion, inflammatory cytokines, melatonin, neurotrophic factors, gut microbiota, oxidative stress, and signal transduction pathways.[7,8] The p38 mitogen-activated protein kinase (p38 MAPK) is part of the MAPK family and plays a role in cell proliferation, differentiation, apoptosis, oxidative stress, and inflammation.[9] When activated by oxidative stress, p38MAPK further activates downstream transcription factors, including nuclear factor κB (NF-κB), which increases the secretion of inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Activation of the p38-MAPK/NF-κB signaling pathway is closely associated with the onset of insomnia.[10,11] Therefore, it is hypothesized that SZS may influence insomnia by inhibiting the activation of the p38MAPK/NF-κB signaling pathway.

This study established an insomnia rat model using intraperitoneal injections of p-chlorophenylalanine (PCPA) and treated the rats with SZS extract and zopiclone to explore the effects of SZS on insomnia. Previous research identified 3.0 g/kg (raw material quantity) as the optimal effective dose of SZS extract for improved insomnia in rats. This study investigated the effect of SZS extract on p38-MAPK/NF-κB signaling pathway to explore the mechanism of SZS in improving insomnia.

Materials

Animals

Forty male SPF-grade Sprague-Dawley (SD) rats, 8 weeks old, with a body mass of 170 to 200 g, were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd [license no.: SCXK (Beijing) 2021–0011]. The rats were housed at the Experimental Animal Center of the Beijing University of Chinese Medicine, and kept in an individually ventilated system at a temperature of 22 to 24°C and relative humidity of 45 to 55%. The rats had free access to food and water. The experiment was approved by the Medical Ethics Committee of the Beijing University of Chinese Medicine (No.: BUCM-2023032803–1139) and followed the "3R" principle of animal welfare.

Drugs and Reagents

The following drugs were used in the study: SZS extract (Academy of Military Medical Sciences, China); zopiclone tablets (Shandong Qilu Pharmaceutical Co., Ltd., China, batch no.: H10980163); PCPA (Shanghai Lohn Chemical Technology Co., Ltd., China, batch no.: 7424–00–21); and sodium pentobarbital (Beijing Chemical Reagent Research Institute, China; batch no.: 060222).

The following reagents were used in the study: BCA protein quantification kit (Beijing Solarbio Science & Technology Co., Ltd., China, batch no.: PC0020); IL-1β, TNF-α, glutathione peroxidase (GSH-Px), carnitine acetyltransferase (CT) enzyme-linked immunosorbent assay (ELISA) kits (Quanzhou Jiubang Biotechnology Co., Ltd, China, product nos.: QZ-10037, QZ-10160, QZ-14723, QZ-14725); p-p38MAPK and p-NF-κBp65 polyclonal antibodies (Aifang Biological, China, product nos.: AFWP1310, AFWP0475); p38MAPK and NF-κBp65 polyclonal antibodies, Glyceraldehyde-3-Phosphate-dehydrogenase (GAPDH) monoclonal antibody (Proteintech Group, USA, product nos.: 14064–1-AP, 10745–1-AP, 60004–1-Ig); goat anti-rabbit IgG (H&L)-HRP conjugated, goat anti-mouse IgG (H&L)-HRP conjugated [Baiao Yijie (Beijing) Technology Co., Ltd, China, catalog nos.: BE0101, BE0102].

Equipment

The following equipment was used in the study: electronic balance (Sartorius, Germany, BSA224S); –20°C refrigerators

(Hefei Kinghome Electrical Co., Ltd., China, BCD-258C); electric hot blast drying oven (Xutemp, China, XT5118L-OV70); vertical electrophoresis system (Bio-Rad, USA, Mini P-4); transmission electron microscope (Hitachi, Japan, HT7800); Synergy II microplate reader (Bio-Tek, USA, 800TS); chemiluminescence and fluorescence imaging system (Clinx Science Instruments Co., Ltd, China, ChemiScope 6100); thermostatic shaker (IKA, Germany, KS 3000 ic); DLAB LED digital display heating metal bath (DLAB Scientific Co., Ltd., China, HB120-S); and desktop freeze centrifuge (Eppendorf, Germany, 5811).

Methods

Modeling and Grouping

After 1 week of adaptive feeding, the rats were randomly divided into the control group (N, $n = 10$) and the modeling group ($n = 30$). The rats in the modeling group received 500 mg/kg of PCPA suspension intraperitoneally for 6 days, with mild stimulation to enhanced modeling effects. The control group received an equal volume of weak alkaline saline. After successful modeling, the rats were randomly divided into the model group (M), zopiclone group (Y, 1.25 g/kg), and SZS extract group (SZS; 3.0 g/kg), with 10 rats in each group. The rats were administered the treatments by gavage for 7 days. The control and model groups received equal volume of distilled water. During modeling and treatment, the condition of the rats' fur, activity level, mental state, food and water intake, and body mass were monitored daily.

Sodium Pentobarbital Coadministration Sleep Test

The rats were injected intraperitoneally with 40 mg/kg of 0.8% sodium pentobarbital solution. Sleep latency was defined as the time from injection to loss of the righting reflex (when the rat's tail naturally droops and limbs show no resistance). Sleep duration was defined as the time from loss to recovery of the righting reflex (continuous spontaneous movement three times). Prolonged sleep latency and shortened sleep duration indicated successful modeling. The sleep test was performed 48 h after the final gavage to assess the effects of SZS extract on sleep latency and duration.

Elevated Plus-Maze Experiment

The rats were placed with their heads facing the open arms at the center of the elevated plus-maze. A camera was used to record the rats' free movement trajectories within the maze for 5 min. Between tests, the arms of the maze were cleaned with medical alcohol before testing the next rat. The SuperMaze software was used to analyze the number of entries into the open and closed arms by the rats.

Open Field Test

The bottom of the open field box was divided into 25 small squares of 20 cm × 20 cm each, with the 9 central squares defined as the central zone and the others as the peripheral zone. The rats were gently placed at the intersection of the diagonals in a 100 cm × 100 cm open field box with a black bottom and blue frame. A camera was used to record the rats' free movement trajectories in the open field for 5 min, during which the number of times the rats reared and groomed was observed and recorded. Between tests, the open field box was cleaned with medical alcohol before testing the next rat. The SuperMaze software was used to analyze the total distance moved by the rats and the frequency of their entries into the central zone.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA kits of rat GSH-Px, CAT, IL-1β, and TNF-α were used to measure the levels of GSH-Px and CAT in the hippocampus and IL-1β and TNF-α in the serum of the rats, using a double-antibody sandwich method. Standard samples and the test samples were added sequentially to the microplate wells coated with antibodies to form antibody-antigen-enzyme-labeled antibody complexes. After thorough washing, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added for color development. The reaction was stopped with stop solution, and the optical density (OD) value was measured at a wavelength of 450 nm using a microplate reader. The concentration of the samples was calculated based on the standard curve.

Western Blot

Total protein was extracted from the cerebral cortex of the rats, and the protein concentration was measured using the BCA protein quantification kit. The amount of sample to be loaded was calculated, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, membrane transfer, blocking, and incubation with specific primary antibodies. The p-p38MAPK, p-NF-κBp65, and p38MAPK antibodies were diluted at a ratio of 1:1,000, the NF-κBp65 antibody was diluted at a ratio of 1:3,000, and the internal reference protein GAPDH antibody was diluted at a ratio of 1:50,000. After overnight incubation, the membranes were washed three times with 1× tris-buffered saline +Tween (TBST). They were then incubated with secondary antibodies for 1 h, washed three times, and developed using the gel imaging system. The gray value of the protein bands was analyzed using Image J software.

Transmission Electron Microscopy

The cerebral cortex samples were prepared for electron microscopy by fixation with glutaraldehyde and osmium tetroxide, washing with phosphate buffer saline (PBS), gradual dehydration with acetone, infiltration with epoxy resin, embedding, ultrathin sectioning, and double staining with uranyl acetate and lead citrate. The ultrastructure of the brain tissue was observed, and images were captured under transmission electron microscope.

Statistical Analysis

SPSS 26.0 software was used for statistical analysis. The data for quantitative measurements were expressed as mean ± standard deviation ($\bar{x} \pm s$). For data with normal distribution and homogeneity of variance, *t*-tests (for comparisons between two groups) or least significant difference (*LSD*) variance analysis (for comparisons among multiple groups)

were used. When variances were unequal among multiple groups, *Dunnett's T3* test was employed. A *p*-value of less than 0.05 was considered statistically significant.

Results

Effect of SZS Extract on the General Condition of Insomniac Rats

Compared with the control group, the rats in the model group exhibited a significant decline in overall responsiveness, sluggishness, poor mental state, coarse and dull fur with noticeable piloerection or alopecia, increased irritability, and reduced food and water intake. These rats also showed significant body mass loss ($p < 0.001$). In contrast, the SZS extract and zopiclone groups showed marked improvement in appearance, with significant increase in body mass compared with the model group ($p < 0.001$), as shown in ►**Fig. 1**.

Effect of SZS Extract on Sleep Status of Insomniac Rats

After modeling, sleep latency in the model group was significantly longer ($p < 0.001$), and the sleep duration was significantly shorter ($p < 0.001$) compared with the control group. The SZS extract and zopiclone groups showed a significant reduction in sleep latency ($p < 0.001$) and extension in sleep duration ($p < 0.001$) compared with the model group, with no significant difference between the two treatment groups, as shown in ►**Fig. 2**.

Effect of SZS Extract on Behavior of Insomniac Rats

In the elevated plus-maze test, there were no significant differences in the number of entries into open and closed arms among the groups ($p > 0.05$). However, in the open field test, the model group showed significant reduction in total movement distance ($p < 0.001$), fewer entries into the central zone ($p < 0.01$), and significant decrease in upright times

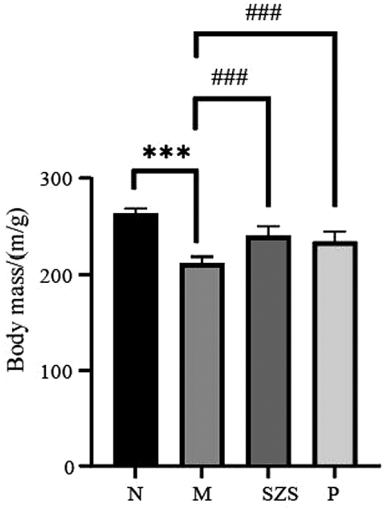

Fig. 1 Effect of SZS extract on the body mass of insomniac rats. Notes: $n = 10$. M, model group; N, control group; P, zopiclone group; SZS, SZS extract group. Compared with the control group, $^{***}p < 0.001$. Compared with the model group, $^{###}p < 0.001$.

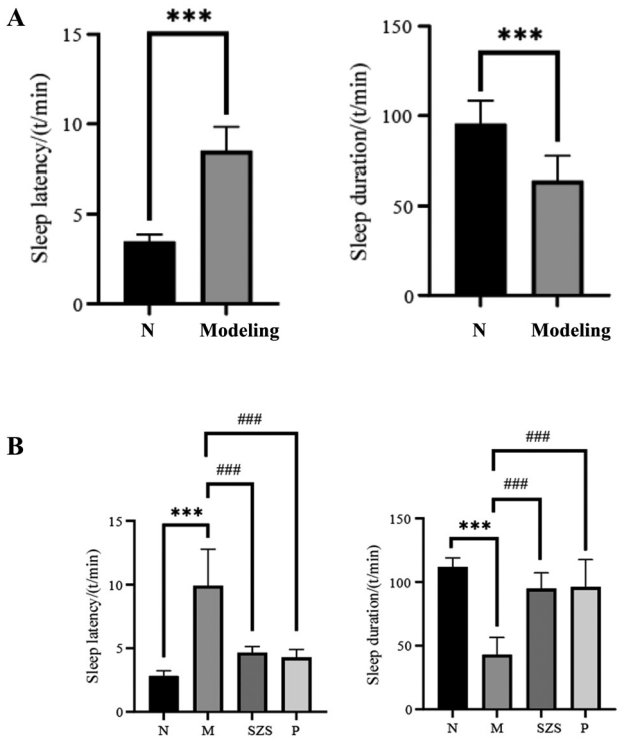

Fig. 2 Effect of SZS extract on sleep status of insomniac rats. Notes: (**A**) The results of sleep latency and sleep duration after modeling. N, control group, $n = 10$. Modeling group, $n = 30$. (**B**) The results of sleep latency and sleep duration after treatment with SZS and zopiclone. $n = 10$. M, model group; N, control group; P, zopiclone group; SZS, SZS extract group. Compared with the control group, $^{***}p < 0.001$. Compared with the model group, $^{###}p < 0.001$.

($p < 0.001$) compared with the control group. The SZS extract group showed significant increase in movement distance ($p < 0.01$) and upright times ($p < 0.001$) compared with the model group, while the zopiclone group also showed a significant increase in these parameters ($p < 0.001$). There was no significant difference in modification times among the groups ($p > 0.05$), as shown in ►**Fig. 3**.

Effects of SZS Extract on Inflammation and Oxidative Stress-Related Indexes in Insomniac Rats

The ELISA results showed that the levels of GSH-Px and CAT in the hippocampus of the model group were significantly lower than the control group ($p < 0.001$). After treatment with SZS extract and zopiclone, the levels of GSH-Px and CAT in the treatment groups were significantly higher than the model group ($p < 0.001$). Additionally, the serum levels of IL-1β and TNF-α in the model group were significantly higher than the control group ($p < 0.001$). After treatment with SZS extract and zopiclone, the levels of TNF-α and IL-1β were significantly reduced compared with the model group ($p < 0.001$), as shown in ►**Fig. 4**.

Effect of SZS Extract on the Proteins Expression Level of p38MAPK/NF-κB Signaling Pathway in Insomniac Rats

Western blot analysis revealed that the proteins expression levels of p-p38MAPK and p-NF-κBp65 in the cerebral cortex of

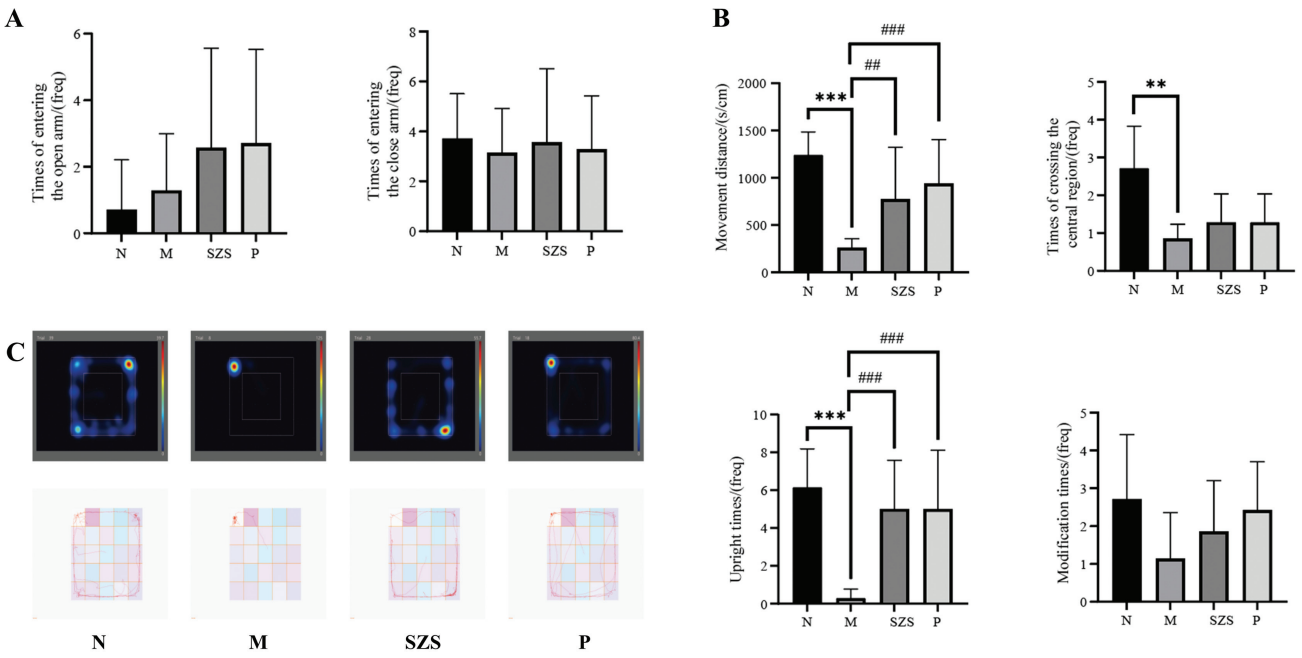

Fig. 3 Effect of SZS extract on the behavior of insomniac rats.
Notes: (**A**) The results of elevated plus-maze test. (**B**) The results of open field test. (**C**) The heat maps and tracks of open field test. $n = 7$. M, model group; N, control group; P, zopiclone group; SZS, SZS extract group. Compared with the control group, $^{**}p < 0.01$, $^{***}p < 0.001$. Compared with the model group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

the model group were significantly higher than the control group ($p < 0.05$). The SZS extract group showed significant reduction in p-p38MAPK levels compared with the model group ($p < 0.05$), while the zopiclone group did not show significant difference in p-p38MAPK levels compared with the model group ($p > 0.05$). Both the SZS extract group and the zopiclone group showed significant reduction in p-NF-κBp65 levels compared with the model group ($p < 0.001$). There was no significant difference in p38MAPK and NF-κBp65 levels among the groups ($p > 0.05$), as shown in ►**Fig. 5**.

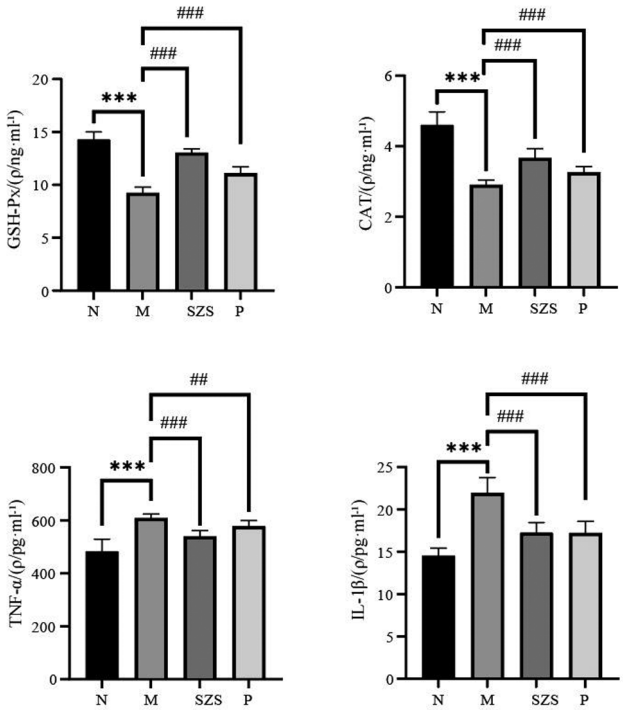

Fig. 4 Effects of SZS extract on inflammation and oxidative stress-related indexes in insomniac rats.
Notes: $n = 7$. M, model group; N, control group; P, zopiclone group; SZS, SZS extract group. Compared with the control group, $^{***}p < 0.001$. Compared with the model group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

Effects of SZS Extract on Neuronal Damage in Insomniac Rats

Transmission electron microscopy revealed that the neuronal nuclei in the control group had clear and intact nuclear membranes, with visible rough endoplasmic reticulum, ribosomes, microtubules, microfilaments, and mitochondria with well-organized cristae and a clear double membrane structure. In contrast, the model group showed significant neuronal degeneration and edema, with chromatin condensation along the nuclear membrane, reduced and disorganized mitochondria, swollen inner chambers with vacuolation, blurred double-membrane structures, and expanded rough endoplasmic reticulum forming large vacuoles. The SZS extract group showed significant improvement in neuronal structure compared with the model group, with more intact nuclear membranes, more evenly distributed chromatin, reduced mitochondrial swelling, and more organized cristae. The zopiclone group, however, did not show significant improvement in neuronal damage compared with the model group, as shown in ►**Fig. 6**.

Discussion

The prevalence of insomnia is increasing yearly and is becoming more common worldwide. Insomnia can also

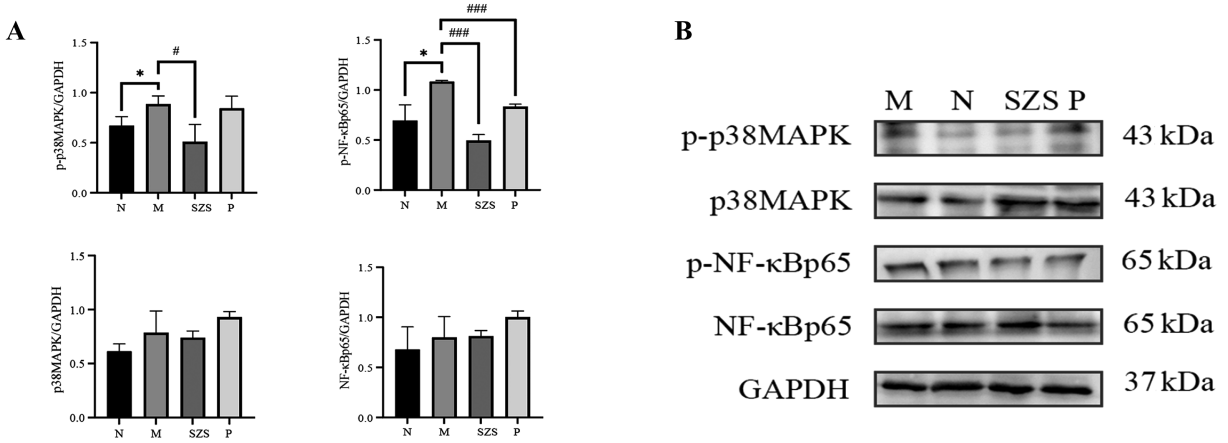

Fig. 5 Effect of SZS extract on the proteins expression level of p38MAPK/NF-κB signaling pathway in insomniac rats.
Notes: (**A**) The comparison of protein band gray values. (**B**) The protein band diagram. $n = 4$. M, model group; N, control group; Y, zopiclone group; SZS, SZS extract group. Compared with the control group, $^{*}p < 0.05$. Compared with the model, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$.

lead to other mental disorders, such as anxiety and depression, greatly impacting patients' daily lives.[12] Therefore, treating insomnia is crucial. The neurotransmitter serotonin (5-hydroxy tryptamine, 5-HT) plays a direct role in regulating the sleep–wake cycle, and decrease in 5-HT levels is a significant pathological basis for anxiety.[13,14] PCPA is an inhibitor of tryptophan hydroxylase, an essential enzyme for 5-HT biosynthesis. By inhibiting tryptophan hydroxylase activity, PCPA reduced 5-HT levels in the brain and serum, and led to sleep–wake cycle disturbances.[15] Consequently, the PCPA-induced insomnia rat model is widely used in insomnia research. In this study, after 6 days of modeling, the rats exhibited disrupted circadian rhythms, increased daytime activity, rough and dull fur with noticeable piloerection, and body mass loss, consistent with the basic characteristics of insomnia. The sodium pentobarbital coadministration sleep test confirmed that sleep latency was significantly prolonged, and sleep duration was significantly shortened after modeling, demonstrating the successful construction of an insomnia rat model.

The complex pathogenesis of insomnia limited the efficacy of typical clinical treatments. The multitarget, multipathway characteristics of TCM offer new avenues for treating insomnia. The active components of SZS extract include saponins, flavonoids, alkaloids, and fatty acids, which have been shown to possess sedative, hypnotic, memory-improving, and anti-anxiety properties.[16–19] This study used zopiclone, a commonly used clinical drug for treating insomnia, as a positive control to explore whether SZS extract could effectively improve insomnia. The results showed that SZS extract significantly shortened sleep latency and extended sleep duration in insomniac rats, with no difference compared with zopiclone. Additionally, the rats treated with SZS extract showed significant body mass gain and improvement in their overall condition. The open field test results indicated that SZS extract improved cognitive and behavioral impairments induced by insomnia and increased the rats' autonomous activity. The transmission electron microscopy results revealed that insomnia caused damage to the neurons in the brain, while SZS extract treatment significantly restored the neuronal structures, suggesting that SZS extract could protect the nervous system by reducing neuronal damage.

Insomnia often induces abnormalities in cellular energy metabolism and decreases the body's antioxidant capacity, and leads to oxidative stress, inflammation, and other biological effects.[20] Studies have shown that sleep deprivation inhibits mitochondrial respiration, causes mitochondrial damage, and increases the production of reactive oxygen species (ROS).[21] If antioxidants cannot promptly clear excess ROS, the redox system's dynamic balance will be disrupted, leading to lipid peroxidation and oxidative stress.[22] Other studies have suggested that oxidative stress damage in the

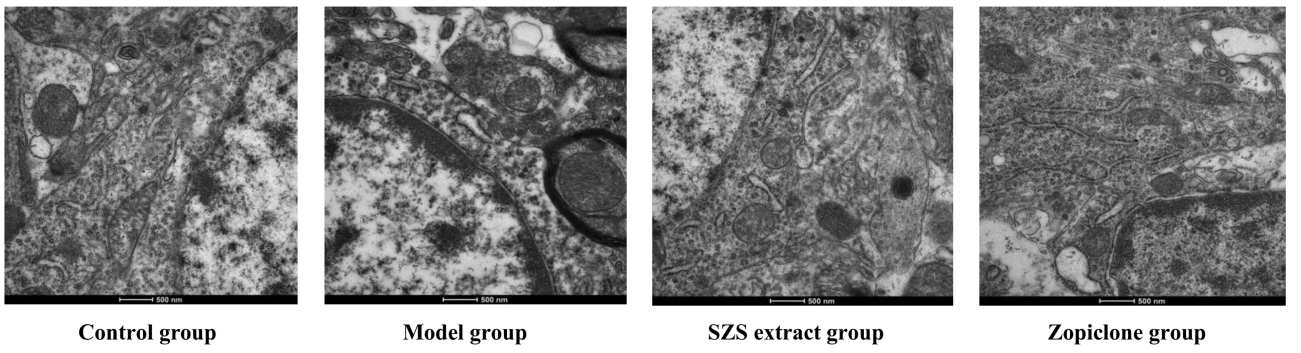

Fig. 6 Effects of SZS extract on neuronal damage in insomniac rats (×22000).

hippocampus may contribute to cognitive or behavioral impairments caused by insomnia.[23] Upon oxidative stress, the MAPK family proteins were activated. The MAPK family includes three subclasses: extracellular signal-regulated kinases (ERK1 and ERK2), p38MAPK (including P38α, P38β, P38γ, and P38σ), and c-Jun N-terminal kinases (JNK1, JNK2, and JNK3).[24] Among them, p38MAPK is a stress-activated proinflammatory kinase that regulates the activation of downstream proinflammatory transcription factor NF-κB.[25–27] When NF-κB is activated, the inhibitory protein IκB undergoes phosphorylation, ubiquitination, and degradation, allowing the active NF-κB dimer to enter the nucleus and bind to DNA, initiating gene transcription and influencing the expression of inflammatory cytokines.[28] The proinflammatory and anti-inflammatory balance is closely related to the normal operation of the sleep–wake cycle.[29] The schematic diagram of the signaling pathway is shown in ►**Fig. 7**. This study found that the levels of the antioxidant enzymes GSH-Px and CT in the hippocampus were significantly lower in the model group, indicating that insomnia reduces these enzymes and disrupts the brain's redox balance, leading to oxidative stress. SZS extract significantly increased the levels of GSH-Px and CT, suggesting that it could effectively enhance the brain's antioxidant capacity and reduce oxidative stress. The model group also showed significantly increased expression of p-p38MAPK and p-NF-κBp65 proteins in the brain compared with the control group, indicating that insomnia activates the p38MAPK/NF-κB signaling pathway. SZS extract significantly reduced the expression of p-p38MAPK and p-NF-κBp65, suggesting that it inhibited the activation of this pathway. Previous studies have shown that insomnia increases the levels of inflammatory cytokines in the peripheral circulation.[30] This study also found that the levels of IL-1β and TNF-α in the serum of the model group were significantly higher than those in the control group, consistent with previous research. The SZS extract group showed significantly lower levels of IL-1β and TNF-α compared with the model group, indicating that SZS extract could reduce the levels of proinflammatory cytokines, possibly by inhibited activation of the p38MAPK/NF-κB signaling pathway.

Conclusion

In summary, SZS extract can effectively improve insomnia, alleviate neuronal damage, and reduce cognitive-behavioral impairments. Its mechanism of action may involve the inhibition of the p38MAPK/NF-κB signaling pathway. This study provided preliminary insights into the molecular biology mechanism of SZS extract in treating insomnia and offered a reference for future research on related mechanisms.

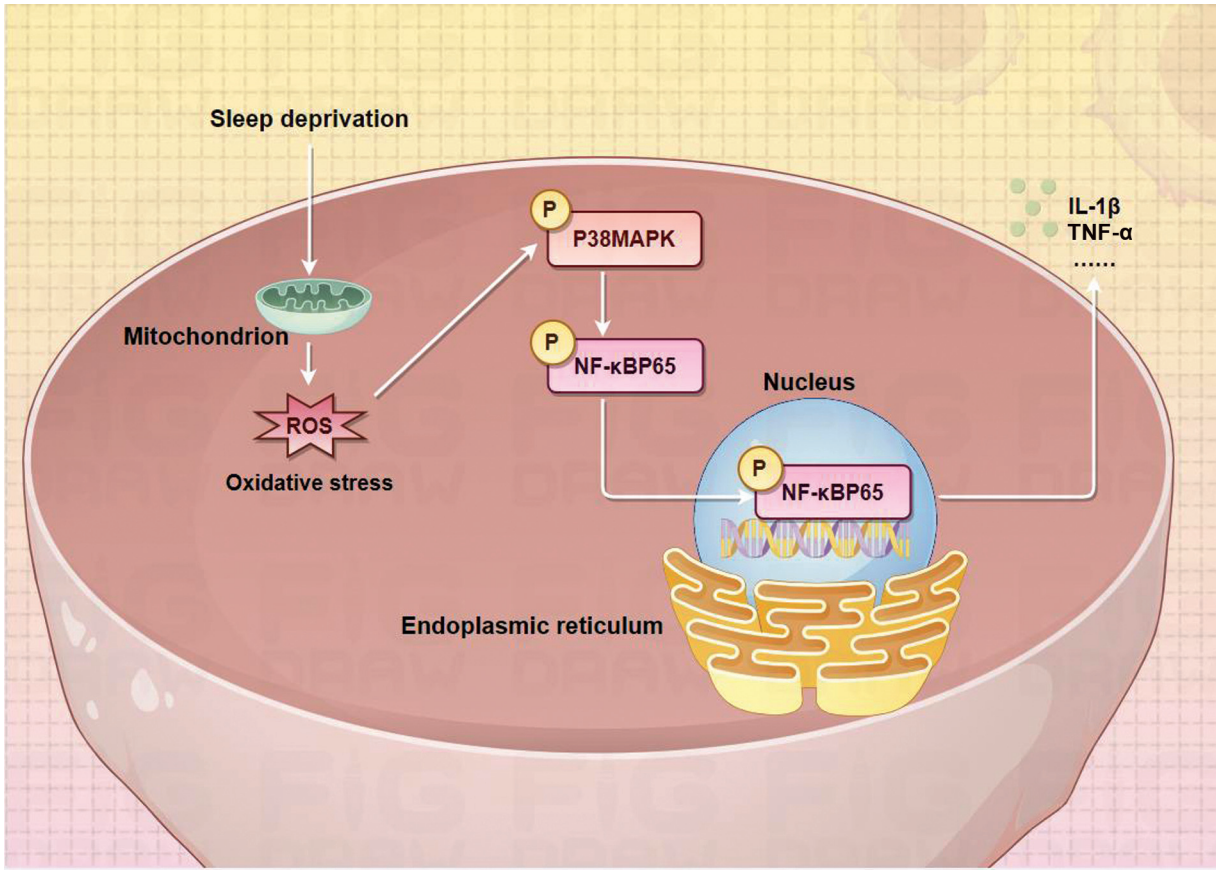

Fig. 7 Schematic diagram of sleep-deprivation triggering oxidative stress and activating the p38MAPK/NF-κB signaling pathway. Abbreviations: IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

CRediT Authorship Contribution Statement

Mingyu Ji: Conceptualization, investigation, data curation, formal analysis, methodology, validation, visualization, writing -original draft. **Wei Xiong, Zijing Xu, Peipei Zhang:** Investigation, data curation, methodology. **Shuyu Li, Qian Wang, Dexian Jia:** Funding acquisition, project administration, resources, supervision, writing-review & editing.

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Conflict of Interest

The authors declare no conflict of interest.

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