

Original Research Article

Chaihu-Longgu-Muli Decoction promotes sleep in mice with insomnia

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Abstract

Purpose: To determine the hypnotic and sedative effects, and the mechanism of action of Chaihu-Longgu-Muli Decoction (CLMD) in mice with insomnia.

Methods: A mice model induced with insomnia using *p*-chlorophenylalanine (PCPA), was established. The sedative and hypnotic effects of CLMD on mice were evaluated by animal behavioral tests. The animals were separated into four experimental groups: (1) control group, (2) PCPA group, (3) PCPA + 5 mg/kg CLMD group, and (4) CPA + 10 mg/kg CLMD group. The 5-HTR1A expression was determined by western blot. The expression levels of the neurotransmitters were assessed using commercial ELISA kits, while the histological changes in hypothalamic tissues were examined microscopically after staining with hematoxylin and eosin (H & E).

Results: CLMD exerted a sedative effect on and ameliorated anxiety-like and stressful behaviors in mice with insomnia. Moreover, CLMD induced sleep and enhanced 5-HTR1A to modulate neurotransmitter secretion. The data also indicate that CLMD exerted a protective role by alleviating hypothalamic damage and regulating the activation of NLRP3 inflammasome associated with insomnia.

Conclusion: These findings indicate that CLMD has sedative and hypnotic effects, and offers a new therapeutic modality for insomnia treatment.

Keywords: Insomnia, Chaihu-Longgu-Muli Decoction, *p*-Chlorophenylalanine, sedative, Hypnotic

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INTRODUCTION

Insomnia is a common clinical sleep disorder that affects people of all ages. Insomnia includes poor sleep quality, difficulty falling asleep, recurrent waking, and short overall sleep duration[1]. Globally, numerous people are affected by mild insomnia, and some suffer from

severe or chronic insomnia. The mechanisms and causes of insomnia are complex and are often associated with drug use and significant medical and psychiatric disorders [2]. However, hypnotic drugs, including cognitive benzodiazepines, benzodiazepine receptor agonists, and antihistamines, produce many adverse side effects in clinical application,

including rebound insomnia, dependency, and drug tolerance.

Traditional Chinese Medicine (TCM) plays a key role in Chinese medicine, as well in the treatment of insomnia. Chaihu-Longgu-Muli Decoction (CLMD) was prescribed by Zhang Zhongjing, a famous Han Dynasty medical practitioner. Currently, CLMD is used in the treatment of neurological diseases, where it may improve hippocampal neuronal pyroptosis by reducing nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) expression [3]. In addition, CLMD is used widely and clinically for the treatment of insomnia, and has been reported to be an effective therapeutic [3].

The NLRP3 inflammasome plays an essential role in innate immunity [4]. Activated NLRP3 recruits and stimulates the pro-inflammatory protease caspase-1 [5]. The precursors of interleukin 1 β (IL-1 β) and IL-18 are cleaved by activated caspase-1, thereby triggering an inflammatory response [6]. The current research showed that NLRP3 expression increased in patients with insomnia [7]. However, the mechanism of action of CLMD for insomnia remains unclear. Therefore, the present study aims to investigate the effects of CLMD on insomnia, and its mechanism of action.

EXPERIMENTAL

Animals

The study used male wild-type ICR mice weighing 20 \pm 2 g, obtained from Weitong Lihua Biology Company in Beijing. All animal experiments were approved by the Animal Ethics Committee of the Ethics Committee of Beijing Luhe Hospital affiliated with Capital Medical University (approval no. 2021-LHKY-095-02), and were conducted in line with international guidelines for animal studies [8].

The animals were divided into four experimental groups of six mice each: (1) Control; (2) PCPA; (3) PCPA + 5 mg/kg CLMD; and (4) PCPA + 10 mg/kg CLMD. To simulate mental stress and insomnia in humans, the mice were subjected to mild, chronic stressors and treated with PCPA. The stressors consisted of various procedures, such as 24 h of food deprivation, 1 min of tail cramping, 24 h of reversal of day and night, exposure to 4 °C cold water, 5 min of swimming, 24 h of water deprivation, 24 h of limb restraint, 24 h of cage-tilting at 45°, and 24 h of wet sawdust bedding. After the chronic and unpredictable mild stress treatment which lasted for 15 to 16 days, the mice were intraperitoneally

injected with PCPA 30 mg/mL at a dose of 300 mg/kg, while the control group was administered an equal volume of distilled water.

Behavioral tests

During the second week of the experiment, an autonomous activity experiment [9] was implemented. After PCPA treatment, the mice were placed in a mouse automatic activity recorder and allowed to acclimatize for 2 min, following which their locomotor activity was detected and analyzed for 5 min.

Three days prior to the end of the experiment, the elevated plus maze (EPM) test [10] was performed. The labyrinth used in the experiment was raised 50 cm above the floor, and consisted of two open arms and two closed arms, each measuring 25 cm, and a central area measuring 5 \times 5 cm. Each mouse was placed in the central area of the open arm and allowed to explore the labyrinth for 5 min while being recorded by a computerized video recording system. The number of entries and the time spent by the mouse in the open arm were calculated as a percentage of the total time spent in the labyrinth.

Two days before the end of the experiment, the open field test (OFT) [11] was performed. The mice were placed in the middle of a 50 cm \times 50 cm surface area field and their behavioral parameters were analyzed for 5 min.

Western blot

Brain tissue was used to extract total protein. After separating the protein samples (60 μ g protein/sample, as determined by a BCA kit) using 8 % SDS-PAGE gel, and transferring them onto nitrocellulose membranes, the membranes were blocked with blocking buffer and subsequently exposed to primary antibodies 5-HTR1A (1:2000, Abcam, Cambridge, UK), NLRP3 (1:5000, Cell Signaling Technology, USA), IL-1 β (1:5000, Santa Cruz Biotechnology, USA), caspase-1 (1:8000, Novus Biologicals, USA), ASC (1:2000, Santa Cruz Biotechnology), IL-18 (1:2000, Novus Biologicals), and GAPDH (1:20000, Novus Biologicals) overnight at a temperature of 4 °C. Afterwards, an enhanced chemiluminescence reagent (Bio-Rad, USA) was used to detect the membranes following a 1-hour incubation with secondary antibody.

Enzyme-linked immunosorbent assay (ELISA)

The neurotransmitters- dopamine (DA), 5-hydroxytryptamine (5-HT), and norepinephrine

(NE) were quantified through ELISA by following the manufacturer's protocols (R&D Systems, USA).

Morphological studies

Hypothalamus tissues were fixed in a 10 % paraformaldehyde solution, embedded in paraffin, and sectioned at a thickness of 4 μ m. The slides were stained with H&E to visualize the pathological changes in the hypothalamus tissues. The grading of these changes was performed using a double-blind method. After H & E staining, pathological changes in the hypothalamus tissues were graded using the double-blind method. A light microscope (Nikon, Tokyo, Japan) was used to capture the tissue images.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 6.0 (GraphPad Software, La Jolla, USA), and the data presented as mean \pm standard error of the mean (SEM). All experiments were performed in triplicate., and one-way analysis of variance (ANOVA) and t-test were used to compare groups as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Sedative effect of CLMD on mice with insomnia

The vertical and horizontal locomotor scores of the PCPA group were higher than those of the control group. The vertical and horizontal movement activities of mice in the PCPA + 5 mg/kg CLMD group and PCPA + 10 mg/kg CLMD group were lower than those of the PCPA group (Figure 1 a, b and e). In addition, the percentage of open arm time (OT %) and the percentage of entries into the open arms (OE %) of mice in the PCPA group were lower, whereas the OT % and OE % of mice in the PCPA + 5 mg/kg CLMD and PCPA + 10 mg/kg CLMD groups showed a dose-dependent increase (Figure 1 b and c). These results suggest that CLMD had a sedative effect that ameliorates anxiety-like and tense behaviors in mice with insomnia.

Effect of CLMD on pentobarbital-induced sleep

To evaluate the effect of CLMD on sleep, a hypnotic dose of pentobarbital sodium (55 mg/kg) was used to induce sleep in the mice with

insomnia, and then sleep latency and duration were measured. Sleep duration was prolonged in mice treated with a hypnotic dose of pentobarbital sodium and CLMD (Figure 2 a). However, sleep latency was extended upon treatment with PCPA, and sleep duration was reduced in the PCPA + 5 mg/kg CLMD and PCPA + 10 mg/kg CLMD groups (Figure 2 b). These results demonstrate that CLMD induced sleep and increased the hypnotic effect of pentobarbital by prolonging sleep duration.

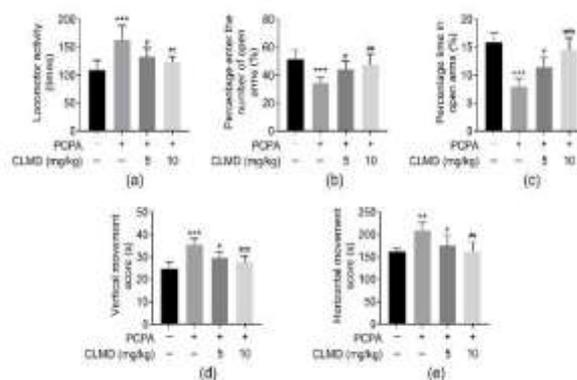


Figure 1: Effects of CLMD treatment on anxiety behavior in mice with insomnia. (A) The number of locomotor activities during the autonomous activity; (B) The percentage of mice entering the open arm during EPM test; (C) The percentage of time spent in the open arm during the EPM test; (D) Vertical movement scores and; (E) Horizontal movement scores were collected during the OFT. Data are presented as mean \pm SD. ** $P < 0.01$, *** $p < 0.005$ compared with the control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.005$ compared with the PCPA group

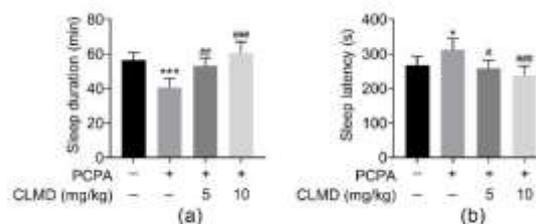


Figure 2: Hypnotic effect of CLMD on pentobarbital sodium-induced sleep-in mice with insomnia. (A) Analysis of sleep duration; (B) Analysis of sleep latency. Data are presented as mean \pm SD. * $P < 0.05$, *** $p < 0.005$ compared with the control group. # p , ## $p < 0.005$ compared with the PCPA group

Effect of CLMD on neurotransmitters in the mice

To identify the regulatory effects of CLMD on monoamine neurotransmitters, DA, 5-HT, NE, and 5-HTR1A expressions were measured in mice with insomnia. The PCPA group showed

higher levels of DA and NE expression, and lower levels of 5-HT expression. In contrast, mice treated with two doses of CLMD exhibited increased 5-HT production and decreased DA and NE expression when compared to the PCPA group (Figure 3 A). The 5-HTR1A, a crucial protein involved in sleep regulation, was also measured by western blot. Its expression was suppressed significantly in the PCPA group compared with the control group, but increased in the PCPA + 5 mg/kg CLMD and PCPA + 10 mg/kg CLMD groups (Figure 3 B). These findings indicate that CLMD ameliorates poor sleep-in mice with insomnia by enhancing the production of 5-HTR1A to modulate neurotransmitter secretion.

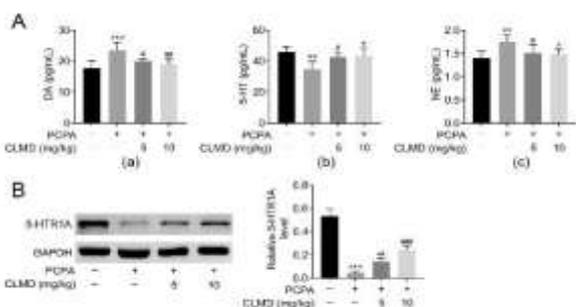


Figure 3: Effect of CLMD on neurotransmitters and 5-HTR1A expression in mice with insomnia. (A) DA levels; (B) The 5-HT levels A; (C) NE levels; (D) 5-HTR1A protein expression. Data are presented as mean ± SD. ***P* < 0.01, ****p* < 0.005 compared with the control group; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.005 compared with the PCPA group

CLMD ameliorated histopathological changes in the hypothalami of mice with insomnia

The protective effects of CLMD on PCPA-induced histopathological changes in the hypothalamus of mice with insomnia were investigated by H&E staining of hypothalamic tissue samples. There was no significant

abnormal pathological change in the hypothalamus tissue of the control group. In the PCPA group, the hippocampal tissue showed abnormal morphology, with vanished neuron shapes and a decrease in Nissl bodies (Figure 4 a and b). However, treatment with CLMD suppressed the PCPA-induced pathologies (Figure 4 c and d). These findings indicate that CLMD mitigated the damage caused by PCPA treatment in the hypothalamus of mice with insomnia.

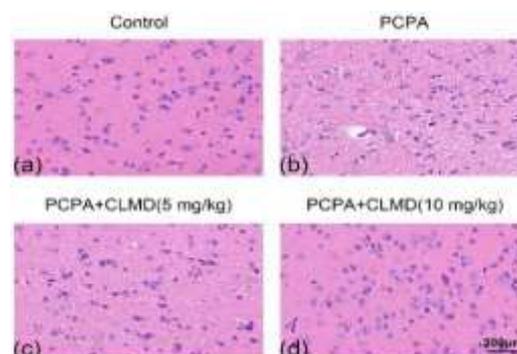


Figure 4: Effect of CLMD on hypothalamic tissue in mice. Histological evaluation of H & E -stained hypothalamic tissue from mice treated with CLMD

Effect of CLMD on activation of NLRP3 inflammasome

To assess insomnia-related activation of the NLRP3 inflammasome, the expressions of NLRP3 inflammasome-related proteins were determined. The expressions of NLRP3, ASC, caspase1, IL-18, and IL-1β were significantly enhanced in the PCPA group and reduced in the CLMD groups (Figures 5 A & 5 B). The data indicated that CLMD plays a protective role by modulating the activation of the insomnia-associated NLRP3 inflammasome.

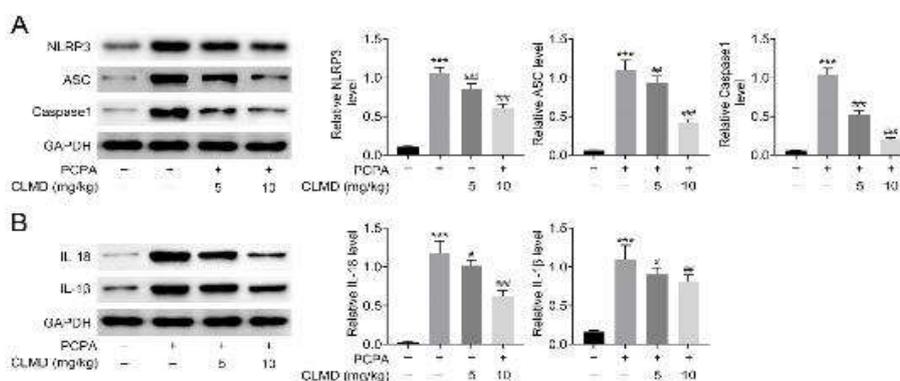


Figure 5: Effect of CLMD on the activation of NLRP3 inflammasome. (A) Protein expression levels of NLRP3, ASC, and caspase 1; (B) Protein expression levels of IL-18 and IL-1β. Data are presented as mean ± SD. ****P* < 0.005 compared with control group; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.005 compared with PCPA group

DISCUSSION

CLMD is a medication that is commonly prescribed to treat insomnia, and various studies have shown that it is both safe and effective for this purpose [12]. Several studies have explored the physiological mechanisms underlying the effectiveness of CLMD in treating insomnia [13]. CLMD has been suggested to potentially regulate monoamine neurotransmitters such as serotonin, DA, and NE in the brain, which could play a role in anxiety and insomnia. However, the exact mechanism of action is still not fully understood. To investigate the sedative and hypnotic effects of CLMD on insomnia, an animal model of insomnia induced by PCPA was utilized. The study included a control group and a PCPA group which did not receive any treatment for insomnia, while two CLMD groups were administered different doses of the medication.

In order to assess the impact of CLMD on autonomous activity as well as the duration and latency of sleep in pentobarbital-induced mice with insomnia, the locomotor activity and the pentobarbital-induced sleep tests were performed. Pentobarbital improved the inhibitory effect of CLMD on the central nervous system by interfering with polysynaptic transmissions in the brainstem [9]. This study demonstrated that CLMD was effective in reducing anxiety and tension-related behavior in mice, with a sedative effect that was evidenced by a decrease in autonomous activity. Furthermore, CLMD was found to have a synergistic effect with pentobarbital in inducing sleep, as it increased the hypnotic effects of pentobarbital sodium by prolonging sleep duration. The mechanism of sleep is known to involve various neurotransmitters, the GABAergic nervous system, and the nitric oxide pathway. Multiple studies suggested that the dysfunction of 5-HT and other neurotransmitters are associated with insomnia [14]. Previous studies showed that 5-HTergic and GABAergic systems in the central nervous system were regulated by therapeutic drugs that induce sedation and hypnosis [15]. 5-HT, also called serotonin, is one of the neurotransmitters that modulate the sleep-wake cycle by suppressing the midbrain reticular activation system or influencing the blue spot's NE component. 5-HTR1A may also suppress the secretion of 5-HT from nerve terminals so as to regulate sleep. Conversely, the tryptophan hydroxylase inhibitor PCPA may deplete 5-HT to cause insomnia. Consistent with previous studies, this study provides evidence that CLMD facilitates sleep in mice with insomnia by enhancing the production of 5-HTR1A to regulate neurotransmitter secretion.

The hypothalamic suprachiasmatic nucleus is known to be involved in sleep regulation, and numerous studies have confirmed that hypothalamic lesions can result in chronic sleep reduction. This study demonstrated that PCPA treatment may induce hypothalamic damage, while CLMD may alleviate the hypothalamic damage caused by PCPA treatment. The NLRP3 inflammasome is known to play an essential role in the pathogenesis of many diseases. Several studies have reported that NLRP3 inflammasome is activated in the chronic sleep deprivation mouse model [16]. In the chronic sleep deprivation mouse model of the present study, NLRP3 inflammasome was also activated. Consistent with previous studies, this study found that PCPA enhanced the activation of NLRP3 inflammasome, while CLMD regulated the insomnia-related activation of NLRP3 inflammasome.

CONCLUSION

These findings indicate that CLMD has sedative and hypnotic effects, and thus can potentially be developed as a novel therapeutic agent for the management of insomnia.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Pei Wang and Min Li designed and carried out the study. Pei Wang, Siyuan Xin, Zheng Wang, Man Yu, Jiao Wang, and Jinfeng Li supervised the data collection, analyzed and interpreted the data. Pei Wang and Min Li prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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