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The Effect of Modified Qiyuan Paste on Mice with Low Immunity and Sleep Deprivation by Regulating GABA Nerve and Immune System

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ABSTRACT

Background

Low immunity and sleep disorders are prevalent suboptimal health conditions in contemporary populations, rendering them more susceptible to the infiltration of pathogenic factors. LJC is obtained by modifying Qiyuan Paste, a traditional Chinese medicine formula for nourishing Yin and blood and calming the mind with a long history. Studies have shown that *Dendrobium officinale* can improve the immune function of sleep deprivation mice. Based on the traditional Chinese medicine (TCM) theory, LJC was prepared by adding *Dendrobium officinale* to Qiyuan Paste Decoction.

Methods

Yin deficiency syndrome indicators such as back temperature and grip strength of mice in each group were measured, and behavioral tests and pentobarbital sodium induced sleep test were performed. Automatic biochemical analyzer, enzyme-linked immunosorbent assay (ELISA) kit and other methods were used to detect blood routine, serum immunoglobulin (IgG, IgA, and IgM), complement (C3, C4, and ACP) and LDH content in spleen, serum hemolysin and delayed hypersensitivity (DTH) levels. The serum levels of γ -aminobutyric acid (GABA) and glutamate (Glu) were detected by high performance liquid chromatography (HPLC). Hematoxylin-eosin (H&E) staining and Nissl staining were used to evaluate the histological changes of hypothalamus tissue. Western blot and immunohistochemistry were used to detect the expression of GABA pathway proteins GABRA1, GAD, GAT1, and GABAT1, and the expression of CD4⁺ and CD8⁺ proteins in thymus and spleen tissues.

Results

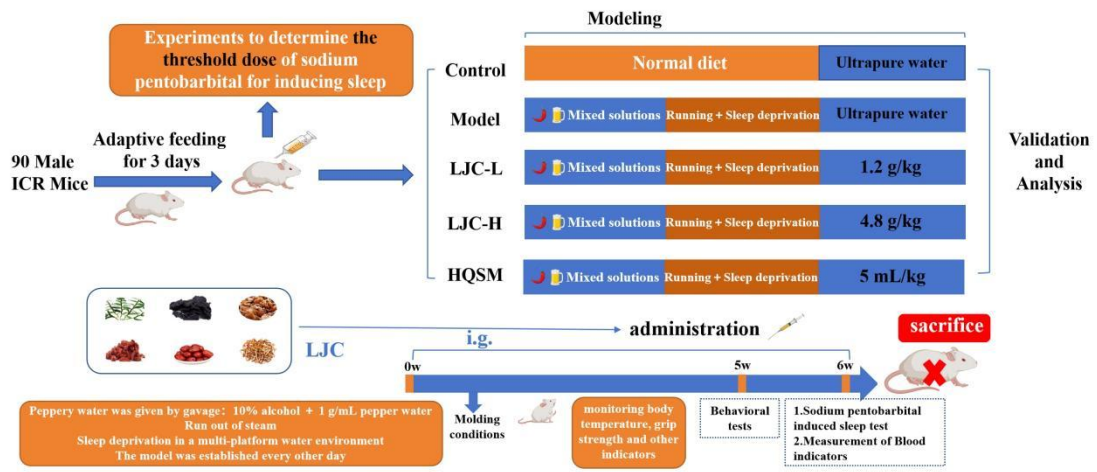
Of note, LJC could prolong the sleep duration, improved the pathological changes of hippocampus, effectively up-regulated GABA content in the brain of mice, and down-regulated Glu content and Glu/GABA ratio, and increased the expression of GABRA1, GAT1, and GAD and decreased GABAT1 expression to improve sleep disorders. Importantly, LJC could improve the damaged thymus and spleen tissues of

the model mice, and increase the activities of ACP and LDH in the spleen of the immunocompromised mice. At the same time, serum hemolysin levels, serum immunoglobulin IgG, IgA, and IgM levels increased after LJC administration, manifested as increased CD4⁺ content, decreased CD8⁺ content, and enhanced delayed-type hypersensitivity (DTH) response. In addition, LJC significantly increased the levels of complement C3 and C4, increased the number of white blood cells and lymphocytes in blood, and decreased the percentage of neutrophils.

Conclusions

LJC can improve the model of immunocompromised mice with insufficient sleep. The mechanism may be to regulate the content of GABA/Glu and the expression levels of GABA metabolism pathway related proteins in the brain of mice, and enhance the specific and non-specific immune functions of mice.

Graphical abstract



KEYWORDS: Modified Qiyuan Paste(LJC); Low Immunity; Sleep Deprivation; GABA; Nervous immune system

1. Introduction

Modern immunology believes that immunity is a physiological function of the human body, and the human body relies on this function to identify "oneself" and "non-self" components, so as to destroy and reject antigenic substances entering the human body or damage cells and tumor cells generated by the human body itself, so as to maintain the physiological balance and health of the body ^[1-4]. When the immune function declines, the body is in a sub-health state and is more susceptible to the invasion of pathogenic factors ^[5-7]. With the rapid advancement of society, there has been a significant increase in individuals facing various issues such as imbalanced dietary patterns, excessive workloads, heightened physical and mental stress levels, and insomnia. The prevalence of low immunity has become a widespread societal phenomenon attributed to factors like unhealthy lifestyle habits. The WHO survey found that 75% of the world's population is in a state of sub-health, and there are a large number of sub-health people such as low immunity and sleep disorders ^[8]. In recent years, it has been discovered that the body's immune function is closely intertwined with sleep quality ^[9-10].

The act of sleeping is a recurring physiological state characterized by physical rest and reduced consciousness, which serves multiple functions, including the enhancement of immune defenses ^[11]. The immune system in human body is composed of immune organs (bone marrow, thymus, spleen, lymph nodes, etc.), immune cells (lymphocytes, mononuclear phagocytes, neutrophils, basophils, eosinophils, mast cells, platelets, red blood cells, etc.), and immune molecules (complement, immunoglobulin (Ig), cytokines). In a normal sleep-wake cycle, immune cell numbers, function, proliferation, and production of immune molecules have their own circadian rhythms: The number of natural killer cells (NK) and neutrophils peaked at noon and reached its lowest point at night. Mononuclear cells, T-spiral cells (CD4+), cytotoxic T cells (CD8+), activated T cells (HLA-DR+), and B cells (CD19), however, reached their maximum value late at night, then decreased during the rest of the night and reached their minimum value in the morning ^[12-15]. Ruiz ^[16] et al.

validate this hypothesis in skin transplanted from mice, showing a redistribution of immune cells during sleep to the spleen and lymph nodes, in contrast to sleep deprived mice. More recently, a study showed that sleep restriction of five hours for one week may lead to decreased phagocytosis and NADPH oxidase activity in neutrophils and a decrease in the levels of CD4⁺ T cells ^[17]. In addition, lack of sleep will also affect the level of immune molecules in the body, resulting in changes in the body's C3a, IgA and other content ^[18]. There is growing evidence that there is a two-way relationship between sleep deprivation and the immune system, that activation of the immune system can affect sleep, while sleep also has an impact on the immune system ^[19-23].

There are multiple factors that modulate sleep and immune responses, and GABA/Glu might be two of the main molecules among these factors. GABA and Glu are extremely high in the brain, especially in the hypothalamus, where about 30% of synapses are γ -aminobutyric acid transmitters ^[24]. GABA is the major inhibitory neurotransmitter in the CNS ^[25], which is formed by the removal of carboxyl group by Glu under the action of glutamate decarboxylase (GAD), and its degradation is performed by γ -aminobutyric acid aminotransferase (GABAT). GABA is released from the presynaptic membrane and binds to GABA receptors in the postsynaptic membrane under the action of specific GABA transporters (GATs) to exert inhibitory effects ^[24,26]. When GABA content increases, the amount of slow wave sleep will be deepened to a certain extent. Studies have shown that GABA transporter subtype 1 (GAT1) has a high affinity with GABA and plays an important role in sleep homeostasis ^[27]. In the human body, there are three types of GABA receptors, namely GABAA, GABAB and GABAC. Among them, GABAA is the most abundant and most important receptor in the brain, belonging to ligand-gated chloride channel protein ^[25,28,29]. Researchers found that the expression of GABAA receptor α 1 subunit (GABRA1) in the hypothalamus of insomnia model animals decreased ^[30-32]. Glutamate is a major excitatory neurotransmitter, and low levels of glutamate can cause fatigue and reduced brain activity ^[33,34]. Sleep deprivation can cause a series of disorders of neurotransmitters in the brain, affect the sleep-wake cycle regulation, and

thus change the level of immune cells and immune molecules in the human body ^[35].

Western medicine is mainly symptomatic treatment in the occurrence and development of diseases, and rarely plays a preventive role, while traditional Chinese medicine is good at treating diseases and has little toxic and side effects. Qiyuan paste, derived from the "Secret Anatomy of Health Preservation", is a time-honored traditional Chinese herbal remedy renowned for its efficacy in nourishing yin and blood, tranquilizing the mind, and enhancing cognitive function. A number of studies have pointed out that *Dimocarpus longan* Lour. and *Lycium barbarum* L. have the function of enhancing immunity and improving sleep ^[36-43]. There are similarities between low immunity and "deficiency syndrome" in the concept of Chinese medicine. According to the theory of TCM, *Dendrobium ferruginum* nourishes Yin and clearing heat, which can effectively improve the syndromes related to "Yin deficiency" ^[44]. In addition, studies have shown that *Dendrobium officinale* can significantly improve cellular and humoral immune functions and enhance immunity ^[45-47]. Therefore, LJC was prepared by adding *Dendrobium officinale* to Qiyuan paste recipe based on traditional Chinese medicine theory. LJC is a formulation consisting of six traditional Chinese medicines, namely *Dendrobium officinale*, *Dimocarpus longan* Lour., *Lycium barbarum* L., *Polygonatum sibiricum* Redouté, *Ziziphus jujuba* Mill. and *Citrus aurantium* L. This herbal blend exerts nourishing effects on yin and promotes circulation while also possessing calming properties for the mind and strengthening the spleen. Consequently, it effectively enhances immunity and improves sleep quality.

Based on the theory of traditional Chinese medicine and previous studies, this experiment was conducted by simulating the unhealthy lifestyle of "improper diet, erratic living and spiritual loss" of human beings, and established an animal model of anthropomorphic immune deficiency with sleep deprivation on mice given peppery water, sleep deprivation in a multi-platform water environment and exhausted running ^[48-51].

Based on previous studies, We hypothesize that LJC may modulate the expression of the GABA signaling pathway in the neuro-immune system, exerting an

influence on immune organs, immune cells, and immune molecules within the body. This modulation could enhance immune response, thereby ameliorating sleep disorders and bolstering overall immunity. Therefore, in this study, we established a mouse model of low immunity with sleep deprivation, and further evaluated the pharmacodynamics of LJC by H&E staining and immunohistochemistry. We determined the contents of immunoglobulin, complement, GABA/Glu and the expression of related pathway proteins to clarify the potential mechanism of LJC to enhance immunity and improve sleep.

2. Materials and methods

2.1 Chemicals and Regents

Dendrobium officinale Kimura et Migo. (20220928) was purchased from Zhejiang Senyu Co., Ltd (Zhejiang, China). *Dimocarpus longan* Lour. (220701), *Lycium barbarum* L. (220601), *Polygonatum sibiricum* Redouté (210303) and *Citrus aurantium* L. (20221119) was purchased from Zhejiang Chinese Medicine University Chinese Medicine Yinpian Co. Ltd (Zhejiang, China). *Ziziphus jujuba* Mill. (20220101) was purchased from Zhejiang Zuoli Baicao Pharmaceutical Co., Ltd (Zhejiang, China). HQSM (20220509) was purchased from Zhejiang Xinguang Pharmaceutical Co., Ltd (Zhejiang, China). H&E dye solution (J22D9Y78310) was purchased from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Chicken red blood cell (SPF grade) (221107) was purchased from Guangzhou Hongquan Biotechnology Co., Ltd (Guangzhou, China). Sodium pentobarbital (P258703) purchased from Chengdu Huaxia Chemical Reagent Co., Ltd (Chengdu, China). Acid phosphatase (ACP) test kit (20221115), LDH lactate dehydrogenase kit (20221107), DAB color development kit (20×) (20221128) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Toluidine blue (BCBW0650) from Sigma. IgA kit (11/2022), IgM kit (11/2022) and IgG kit (11/2022) were purchased from Shanghai Enzyme Linked Biotechnology Co., Ltd (Shanghai, China). Igg-two-step immunohistochemical kit (17E06D1902) was purchased from Bode Bioengineering Co., Ltd. The complement C3 kit (60153325) and complement C4 kit

(60153370) were purchased from Desai Diagnostic Systems Co., Ltd. L-glutamic acid standard (1112G024), γ -aminobutyric acid standard (1102D022) purchased from Beijing Solaibao Technology Co., Ltd. CD4⁺ primary antibody (HF1116), CD8⁺ primary antibody (HG0806) purchased from Hangzhou Hua 'an Biotechnology Co., Ltd. GAD primary antibody (No.20746-1-AP), GAT1 primary antibody (No.20298-1-AP) and GABRA1 primary antibody (No.12410-1-AP) were purchased from Proteintech Group. GABAT1 primary antibody (YT1819) was purchased from Immunoway.

2.2 Drug Preparation

According to the preliminary study, the six Chinese herbs in the LJC compound were soaked in distilled water, heated and reflow for 2 h. The high concentration LJC extract (crude drug concentration 0.48 g/mL) was prepared by rotary evaporator, and then the high concentration extract was diluted with distilled water to obtain the low concentration LJC extract (0.12 g/mL). HQSM was prepared with a final concentration of 0.5 mL/mL suspension, and the daily dosage of mice was 5 mL/kg. The preparation method of pepper water for modeling is to break the dried pepper, extract for 2 hours, concentrate the extraction solution, add anhydrous ethanol, adjust the volume to the ethanol concentration of 10%, so that the concentration of the pepper liquid is 1 g/mL containing raw drug.

2.3 The active components of LJC extracts were analyzed by HPLC

The composition analysis of LJC was performed on an Agilent 1260 infinity HPLC system. The analytical column was Welch Ultimate LP C18 (4.6×250 mm, 5 μ m) at a temperature of 30°C. The flow rate was 1 mL/min, and the injection volume was 10 μ L.

Polysaccharide components in LJC: The extract of LJC was stored in cold storage at 4°C after adding absolute ethanol. After collecting the precipitate, it was put into a vacuum freeze dryer (temperature -50°C, vacuum 0.09 MPa) to dry. When removed, crude polysaccharide of LJC extract was obtained. The resulting crude polysaccharide was then prepared by trifluoroacetic acid hydrolysis and PMP

derivatization. The mobile phase was 0.025 mol/L potassium dihydrogen phosphate solution (A) and acetonitrile (B). The detection wavelength was 250 nm. The elution gradient was 0.00-9.00 min with 17%-20% B, 9.01-18.00 min with 20%-22% B, 18.01-26.00 min with 22%-25% B, 26.01-35.00 min with 25%-30% B, 35.01-40.00 min with 30%-37% B, and 40.01-50.00 min with 37%-50% B.

Non-polysaccharide components in LJC: the LJC extract was dried in a vacuum freeze dryer, the lyophilized powder was removed and added to methanol for ultrasound and filtration, then evaporated in a water bath, and then added methanol to redissolve for later use. The detection wavelength was 210 nm. The elution gradient was 0.00-20.00 min with 5%-10%B, 20.01-30.00 min with 10%-15% B, 30.01-35.00 min with 15%-18%B, 35.01-40.00 min with 18%-22% B, 40.01-46.00 min with 22%-28% B, 46.01-50.00 min with 28%-30% B, 50.01-60.00 min with 30%-35% B, 60.01-75.00 min with 35%-55% B, and 75.01-80.00 min with 55%-65% B.

Preparation of Reference Substances: The glucose, rhamnose, arabinose, mannose, galactose, glucuronic acid, glucosamine, ribose and xylose reference materials were weighed carefully, and methanol was added to make a solution of 1 mg/mL. Precision weighing vitenine 1, vitenine 2, naringenin, naringin, rutin, chlorogenic acid, quercetin, luteolin, hesperidin control substance amount, precision weighing, adding methanol to make a solution of 1 mg/mL.

2.4 Animals and Treatment

ICR male mice weighting 20 ± 2 g were obtained from Hangzhou Qizhen experimental animal Technology Co., Ltd. The animals (SCXK(Zhe)2022-0007) were maintained at a constant room temperature of 22-26°C and a humidity of 50-70% for 12 hours light/dark cycles. They were adaptively given food and water for 3 days. All experiments were performed complied with the Regulation of Experiment Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China. The experiment was received approval by ethics committee of Zhejiang University of Technology (20221125Abzz0100999262).

Before the experiment, 90 male ICR mice were randomly divided into 6 groups

(n = 15) with sodium pentobarbital dose of 55, 50, 45, 40, 35, and 30 mg/kg, respectively. After intraperitoneal injection of the corresponding dose of sodium pentobarbital in each group, Sleep latency time of mice within 20 minutes (from the time of injection of pentobarbital sodium to the time of disappearance of righting reflex) and the duration of sleep of mice (from the time of disappearance of righting reflex to recovery) were recorded, and the dose of 100% sleep of mice without too long sleep time was determined as the above threshold dose of pentobarbital sodium. The subthreshold dose of pentobarbital sodium in 80-90% mice whose righting reflex does not disappear is subthreshold dose.

Then 50 male ICR mice were randomly divided into 5 groups (n = 10). They were divided into control group, model group, LJC-H group (4.8 g/kg, LJC high dose), LJC-L group (1.2 g/kg, LJC low dose), and HQSM group (5 mL/kg). The method of drug administration was adopted in this experiment. In addition to the control group, the other groups were given gavage to make thermotropic drugs in odd days (the dose for the first two weeks was 1.6 g/kg, and the adjusted dose was 5 g/kg later), and underwent exhaustive running training (the adaptive running training was conducted for one week, and the final running time was 30 minutes and the running speed was 25 m/min). Even days, sleep deprivation was performed in a multi-platform water environment (12 hours of sleep deprivation for the first two weeks, then adjusted to 18-21 hours). The control group and model group were given the corresponding volume of normal temperature water by intragastric administration, and the other groups were given the corresponding therapeutic drugs by intragastric administration of 0.1 mL/10 g, once a day, for 6 weeks. Then, blood was collected through the orbit and after the mice were sacrificed, the brain tissue, spleen tissue and thymus tissue were collected and stored in the refrigerator at -80°C.

2.5 Indicators of “Yin deficiency” syndrome

The dorsal region of the mice was captured using a thermal camera, and the temperature was subsequently calculated utilizing FLIR ONE software. Rectal thermometry was employed to measure and record the anal temperature of the mice.

275 Additionally, each mouse underwent a grip strength test where it was pulled back at a
276 predetermined speed until releasing its claw, allowing for recording of maximum grip
277 force. Saliva flow rate in mice was assessed by cutting filter paper into small pieces
278 which were then inserted into their mouths using tweezers. After 5 seconds, the dry
279 weight and wet weight of the filter paper were measured, enabling calculation of
280 saliva flow rate as an increase in weight per second.

281 ***2.6 Pentobarbital sodium-induced sleep Tests***

282 The pentobarbital sodium-induced sleep test in mice is a commonly used
283 behavioral method to assess whether a drug has sedative-hypnotic activity [52]. A
284 pentobarbital sodium induced sleep test was performed 2 days before the end of
285 administration. 1 hour after the gavage, the mice were intraperitoneal injection of
286 pentobarbital sodium (the upper threshold dose was determined to be 50 mg/kg in the
287 pre-experiment), and the sleep duration of mice was immediately recorded.

288 ***2.7 Behavioral Test***

289 Autonomous activity experiment in the 5th week of the experiment [53]. 30
290 minutes after administration, the mice of each group were placed into multifunctional
291 mouse automatic activity recorder to acclimate for 2 minutes, and then the locomotion
292 activity of each mice was measured and collected within 5 minutes.

293 Elevated plus maze (EPM) was performed on mice in the 5th week of the
294 experiment [54]. The labyrinth is composed of two open arms and two closed arms.
295 The arm length is 25 cm, and the central area is a square grid of 5×5 cm. The EPM is
296 elevated 40 cm above the floor. During the test, the mice were placed face to face into
297 the central area of the open arm, and the activities of the mice were filmed with a
298 camera for 5 minutes. The percentage of time and the times of the mice entering the
299 open arm were counted in the later period.

300 Open-field test (OFT) in the 5th week of the experiment [55]. After the drug
301 administration for 30 minutes, mice in each group were subjected to an open-field test.
302 The open field is composed of 40×40×40 cm cartons, and the bottom form is 5×5
303 lattice. During the test, the mice were placed in the center of the open field box, and

the activities of the mice for 5 minutes were recorded by the camera, and the number of cells passed by the mice (recorded as horizontal scores) and the number of times of standing (recorded as vertical scores when the two front feet were lifted or attached to the wall of the box) were counted. The software EthoVision XT17 was used to calculate the moving distance and moving speed, and the track chart of the mice's open field activities was drawn.

2.8 The detection of blood indexes and other immune indicators

The level of hemolysin production and delayed hypersensitivity (DTH) can reflect the immunomodulatory ability of the body [56]. The mice in each group were intraperitoneally injected with 5% chicken red blood cells for 4 consecutive days, with an injection dose of 0.2 mL per mouse. 1 hour after the final administration, orbital blood was collected and the supernatant was obtained through centrifugation. After centrifugation, 10 μ L of serum was taken and diluted 100 times with normal saline. Then, 0.5 mL of 5% chicken red blood cells and 0.5 mL of fresh guinea pig serum (10%) were added to the diluted serum. After mixing, the reaction was stopped by incubating at a water bath temperature of 37°C for 60 minutes followed by an ice bath treatment. Subsequently, centrifugation (2000 r/min, 10 min) was performed to separate the supernatant from the mixture. In the blank control group, normal saline was used as a replacement for serum samples. The absorbance at a wavelength of 540 nm was measured.

Additionally, after 4 days, the initial thickness of the left foot was measured using a vernier caliper three times on average. Subcutaneous injection of 50% chicken red blood cells (20 μ L) took place at the same measurement site. After a period of 24 hours had passed since injection, another measurement using a vernier caliper 3 times on average determined the thickness change in the left foot as DTH response data.

At week 6 of dosing, mice in each group were water deprived for 12 hours, and orbital blood was collected through EDTA anticoagulant tubes. The white blood cell count and the proportion of white blood cells in the blood of mice were determined by automatic hematology analyzer.

Before the end of the experiment, the blood was collected from the orbit of mice and incubated in Ep tube at 37°C for 30 minutes. The blood was centrifuged twice (3600 r/min, 10 min) and the upper serum was collected. Enzyme-linked immunosorbent assay (ELISA) was used to detect the content of immunoglobulin (IgG, IgA, and IgM) in serum according to the instructions. The levels of complement (C3 and C4) in serum of mice were detected by automatic biochemical analyzer.

At the end of the experiment, the spleens of mice was weighed, 9 times the volume of normal saline was added, and ground by a high-throughput tissue grinder. After centrifugation (3500 r/min, 10 min), the supernatant was taken, and the protein concentration in the spleen was determined.

2.9 Histological evaluations

H&E staining was used for the histopathological examination of the thymus and spleen, and the histopathological examination of hypothalamus tissue was evaluated by H&E and Nissl staining. Thymus, spleen and brain tissues were fixed with 4% formalin solution, dehydrated in different concentration of alcohol, and embedded in paraffin. After that, they were cut into 4 µm paraffin section and stained by H&E [57]. At the same time, the number of Nissl bodies was determined by Nissl staining. Paraffin embedded hippocampus sections were cut into 4 µm sections, and then the sections were stained with toluidine blue water solution at 50-60°C for 10 minutes to observed Nissl bodies in nervous cells [58].

2.10 HPLC analysis of γ -GABA and Glu

Same as the previous study [59]. Firstly, 0.5 mL of perchloric acid (0.4 mol/L) was added to 20 µL serum (diluted 5×) and centrifuged at 3000 rpm/min for 15 minutes to remove the protein. Then, 50 µL supernatant was added to 125 µL Na₂CO₃ (1 mol/L) to prepare the sample for HPLC detection. The derivatization reaction was carried out with 200 µL of sample and 400 µL of o-phthalaldehyde (OPA) (5 mg OPA was dissolved in 100 µL of methanol solution, 5 µL of 2-mercaptoethanol was added, and diluted to 5 mL with borate and 0.4 mol/L sodium hydroxide buffer). The contents of amino acids γ -GABA and Glu were determined by HPLC. An EC-C18 column (4.6

mm×150 mm, 4 μm) was used as the injection column at 30°C with acetonitrile-water (50:50, A) and sodium acetate (0.05 mol/L) as the mobile phase. Variable wavelength scanning UV detector (VWD) wavelength was set at 338 nm. The mobile phase A increased by 2% per minute, and the end was 30 minutes.

2.11 Immunohistochemistry and Western Blot Assay

The expression of GABRA1, GAD, GAT1, and GABAT1 proteins in the brain was evaluated by immunohistochemical staining. The brain paraffin tissue sections were incubated with GABRA1, GAD, GAT1, and GABAT1 antibodies, then the sections were incubated with secondary antibody goat anti-rabbit IgG, the signals were observed by DAB solution and the nuclei were counterstained with hematoxylin. At the same time, the expression of CD4⁺ and CD8⁺ protein in thymus and spleen tissues was detected by the same method. The positive expression showed yellow color under microscope. The results of protein levels were evaluated by detecting the integrated optical density (IOD) in positive area [60].

Western blot was used to detect the expression of GABA, GAD, and GAT1 protein. Brain protein was extracted with radioimmunoprecipitation (RIPA) buffer, and protein concentration was determined by BCA protein assay kit. The total protein (100 μg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. After blocking for 2 hours, the protein was incubated with primary antibody at 4°C overnight and washed with TBST. Horseradish peroxidase conjugated affinity purified goat anti-rabbit IgG (H+L) secondary antibody (1:10000) was added and incubated with the target protein. The results were visualized by chemiluminescence (ECL) Western blot detection system. Image J image analysis software was used to analyze the optical density value of the bands, and the expression level was standardized by the relative expression of the target protein (optical density value of the target protein/optical density value of the internal reference protein β-actin). And then the expression level of the protein was analyzed.

2.12 Statistical Analysis

All results were presented as means $\pm$ standard deviation (SD). Results were statistically evaluated using IBM SPSS Statistics 19.0. Significant differences between groups were determined by a Student's t-test or one-way analysis of variance (ANOVA), the graphs were performed by GraphPad Prism 8.0.

3. Results

3.1 Active component analysis of LJC

Our preliminary experiments determined that the main composition of LJC polysaccharide were mannose, rhamnose, glucosamine, glucose and arabinose, and the non-polysaccharide component were mainly chlorogenic acid, vitenin-2, rutin, naringin, hesperidin and naringenin. The typical chromatograms are presented in **Figure 1**.

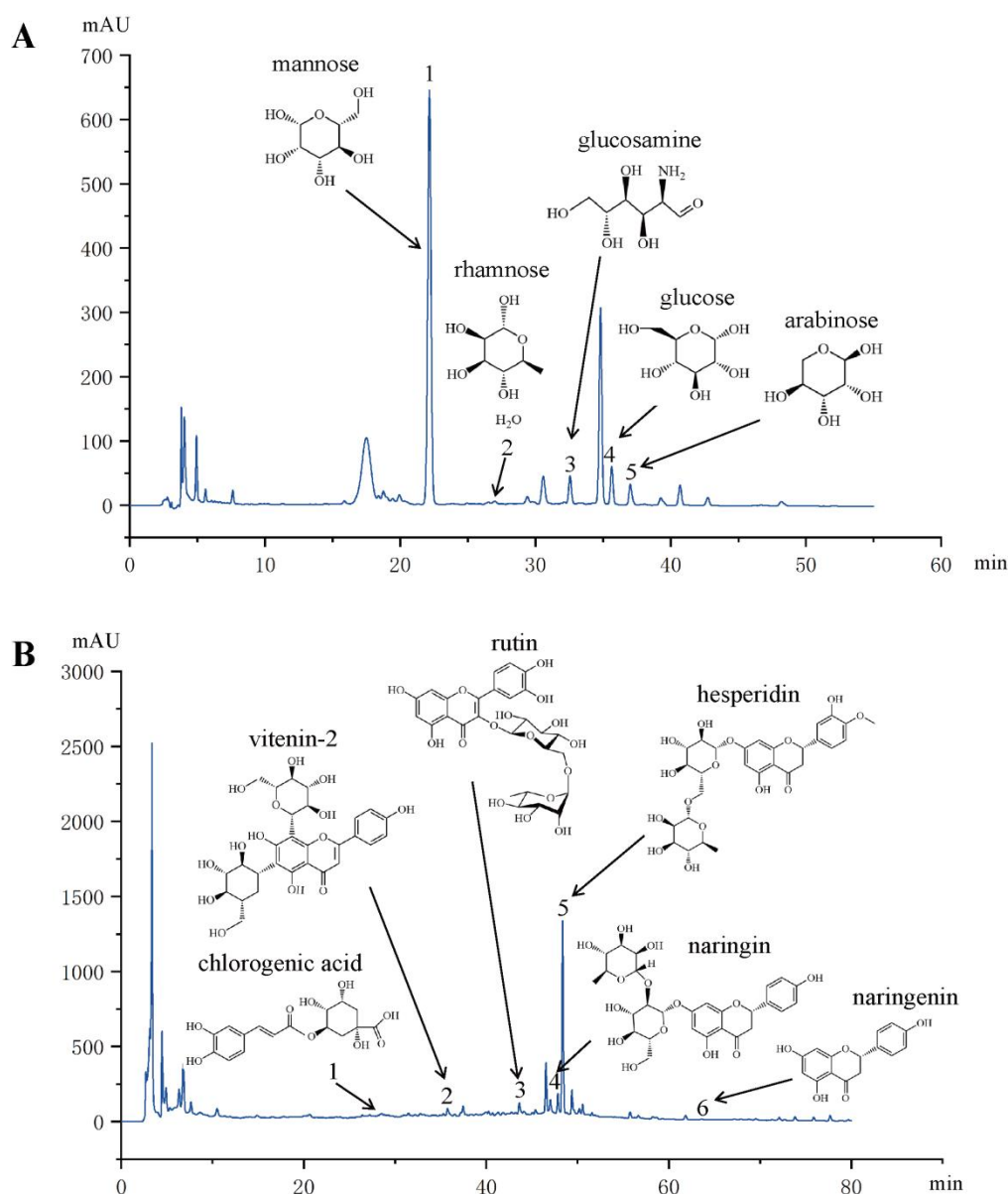


Figure.1. The HPLC profile of the active constituents in LJC. (A) HPLC plot of polysaccharide, 1: mannose, 2: rhamnose, 3: glucosamine, 4: glucose, 5: arabinose; (B) Non-polysaccharide HPLC plot, 1: chlorogenic acid, 2: vitsenin-2, 3: rutin, 4: naringin, 5: hesperidin, 6: naringenin.

3.2 Changes in Body temperature, grip, and saliva flow rate treatment with LJC

In order to evaluate the effect of LJC on Yin deficiency syndrome in mice with low immunity and sleep deficiency, the changes of back temperature, anal temperature, grasping power and saliva flow rate of mice in each group were collected.

Compared to the normal group, mice in the model group gradually exhibited symptoms of "Yin deficiency" after modeling, including increased body temperature, fatigue, and dry mouth. These symptoms were significantly improved in the

medication group. **Table 1** shows the changes of back and anal temperature in mice. After 2 weeks of modeling, compared to the model group, each treatment group showed a significant decrease in back temperature ($P < 0.01$). After 4 weeks of modeling, compared to the model group, only the LJC group demonstrated a significant reduction in rectal temperature ($P < 0.01$). This suggested that LJC can effectively alleviate symptoms related to Yin deficiency and internal heat in modeled mice.

Figure 2A demonstrated that grip strength significantly recovered after LJC and HQSM treatment ($P < 0.01$), with a higher degree of improvement observed in the LJC group than in the HQSM group. This indicated that LJC can effectively improve symptoms associated with Yin deficiency fatigue in modeled mice.

The salivary flow rate index was used to quantify dry mouth symptoms in mice. As shown in **Figure 2B**, all treatment groups exhibited a significant increase in salivary flow rate compared to the model group ($P < 0.05, 0.01$). This suggested that LJC can effectively alleviate dry mouth symptoms in modeled mice.

Table 1 Effects of LJC on back temperature and rectal temperature in mice

Group	Back temperature at week 2(°C)	Rectal temperature at week 4(°C)
NG	28.97±0.90	36.06±0.59
MG	29.73±0.44 [#]	37.07±0.34 ^{##}
LJC-L	28.98±0.64 ^{**}	36.49±0.54 ^{**}
LJC-H	28.97±0.69 ^{**}	36.38±0.71 ^{**}
HQSM	28.69±0.91 ^{**}	36.68±0.65

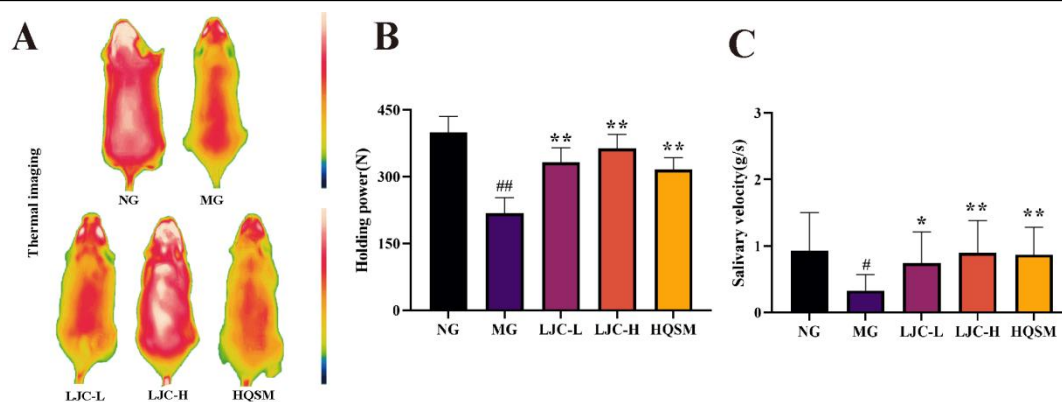


Figure 2. Effects of LJC treatment on body temperature, grip strength, and salivary flow rate. (A) Thermal imaging; (B) Holding power; (C) Salivary velocity. Data are expressed as the mean $\pm$ SD. [#] $P < 0.05$, ^{##} $P < 0.01$ compare with the control group; ^{*} $P < 0.05$ and ^{**} $P < 0.01$ compare with the model group.

3.3 Sedative effect of LJC on insomnia Mice

The effect of LJC on sleep duration induced by a hypnotic dose of sodium pentobarbital (50 mg/kg) was shown in **Figure 3A** after hypnotic dose of sodium pentobarbital mice. Compared with the control group, the sleep duration of the sleep-deprived mice in the model group was significantly shortened ($P < 0.05$). Compared with the model group, the LJC group had a significant increase in sleep duration ($P < 0.05$) significantly enhanced the hypnotic effect of sodium pentobarbital.

The effects of LJC on anxiety and exploratory behavior of sleep deprived mice were evaluated using autonomic activity test, elevated plus maze test, and open field test. The model group exhibited significantly higher autonomic activity compared to the control group ($P < 0.01$). However, the LJC group showed a significant decrease in autonomic activity ($P < 0.05, 0.01$) (**Figure 3B-C**). In the open field test, the model group mice demonstrated significantly increased transverse and longitudinal scores as well as moving distance compared to the control group ($P < 0.05, 0.01$) (**Figure 3D-F**). On the other hand, compared to the model group, both lateral movement scores and moving distance were significantly reduced in the LJC group ($P < 0.05, 0.01$), while only longitudinal scores were decreased in the LJC-H group ($P < 0.05$). As shown in **Figure 3G-H**, the time spent entering into open arms as well as number of entries into open arms were significantly reduced for mice in model groups when compared with those from normal groups ($P < 0.05$). Conversely, both time spent entering into open arms as well as number of entries into open arms for mice treated with LJC-H were markedly increased when comparing with those from model groups ($P < 0.01$). Notably, when comparing with the normal group, irregular and disorganized activity trajectories were observed in the model group; however after treatment with LJC and HQSM, mice in each experimental groups displayed stable and regular activity trajectories similar to those seen in normal mice (**Figure 3I**). Therefore, we concluded that LJC exerted favorable sedative effect on sleep-deprived mouse models.

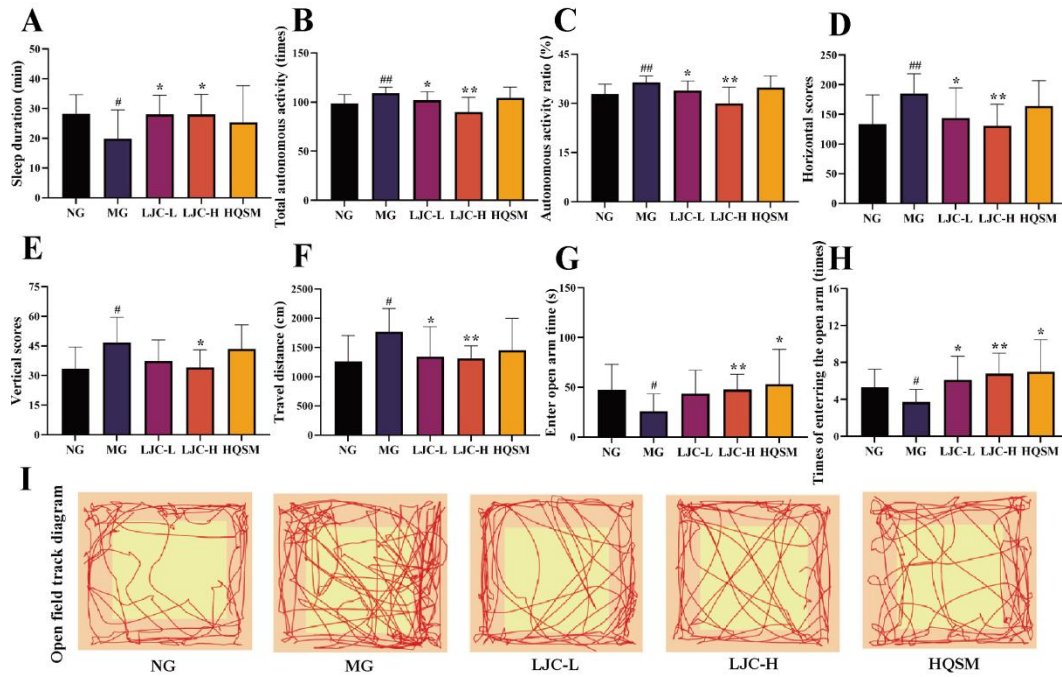


Figure 3. Sedative and hypnotizing effects of LJC in model mice. (A) Sleep duration; (B) Total autonomous activity; (C) Autonomous activity ratio; (D) Horizontal scores; (E) Vertical scores; (F) Travel distance; (G) Enter open arm time; (H) Times of entering the open arm; (I) Open field track diagram. Data are expressed as the mean ± SD. [#] $P < 0.05$, ^{##} $P < 0.01$ compare with the control group; ^{*} $P < 0.05$ and ^{**} $P < 0.01$ compare with the model group.

3.4 The effects of LJC on immune cells and immune molecules

As shown in **Figure 4A-B**, after 4 weeks of treatment, compared with the normal group, the serum hemolysin level and paw swelling degree of the model group mice were significantly decreased ($P < 0.05$); Compared with the model group, the serum hemolysin level of the LJC-H group was significantly increased ($P < 0.01$). In contrast, the paw swelling degree of LJC-H and HQSM groups increased significantly ($P < 0.05$, 0.01). It was suggested that LJC could increase serum hemolysin level and promote DTH reaction.

After 5 weeks of treatment, the number of white blood cells and the percentage of lymphocytes in the peripheral blood of the mice in the model group were significantly lower than those in the normal group ($P < 0.01$), while the percentage of neutrophils was significantly increased ($P < 0.01$); Compared with the model group, the number of white blood cells in the LJC-H group increased significantly ($P < 0.01$). In terms of lymphocyte percentage, both LJC-L and LJC-H groups had varying degrees of increase ($P < 0.05$), and the percentage of neutrophils in LJC-L and LJC-H

groups decreased significantly ($P < 0.05$) (Figure 4C-E). These results suggested that LJC could significantly increase the number of white blood cells in immunocompromised mice with sleep disorders.

Compared with the normal group, the levels of serum complement C3 and C4 in the model group were significantly decreased ($P < 0.05$, 0.01); Compared with the model group, the levels of C3 and C4 in the LJC treatment group were increased to varying degrees ($P < 0.05$, 0.01) (Figure 4F-G). These results suggest that LJC may enhance immunity by activating the complement system.

After 6 weeks of modeling, compared with the normal control group, the serum levels of IgA, IgG and IgM in the model control group were significantly decreased ($P < 0.01$), the levels of IgA, IgG, and IgM were increased in different doses of LJC groups ($P < 0.05$, 0.01). It was suggested that LJC could improve the immunity by stimulating B lymphocytes to secrete antibodies. The results are shown in Figure 4H-J.

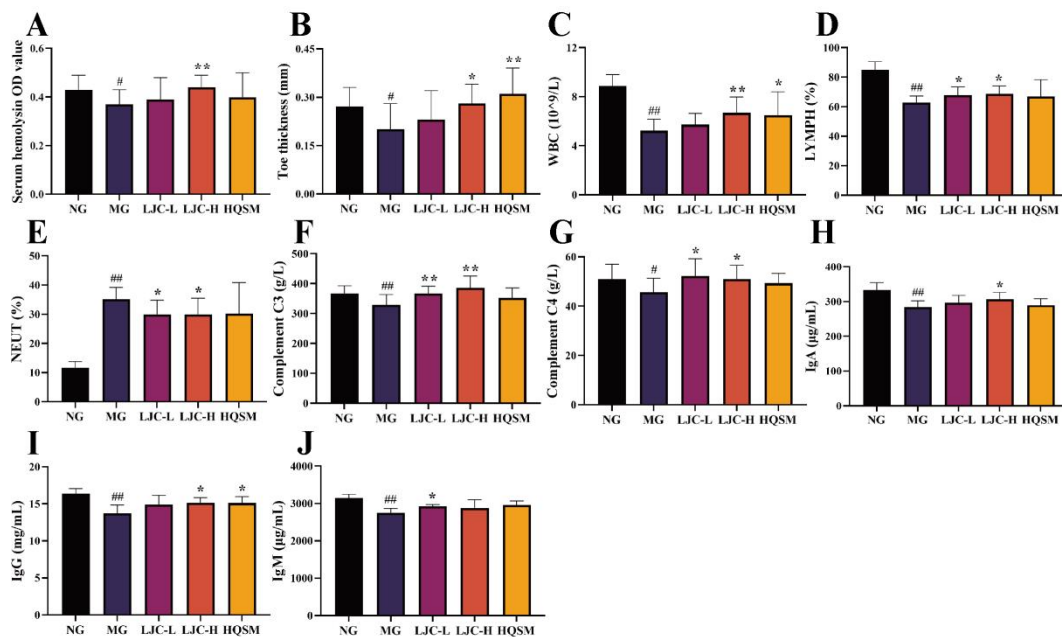


Figure 4. Effects of LJC on immune cells and immune molecules in the blood. (A) Serum hemolysis levels; (B) Toe thickness; (C) Peripheral white blood cell count; (D) The percentage of peripheral blood lymphocytes; (E) The percentage of neutrophils in peripheral blood; (F) Serum complement C3 level; (G) Serum complement C4 level; (H) Serum IgA level; (I) Serum IgG levels; (J) Serum IgM levels. Data are expressed as the mean $\pm$ SD. # $P < 0.05$, ## $P < 0.01$ compare with the control group; * $P < 0.05$ and ** $P < 0.01$ compare with the model group.

3.5 Histopathological evaluation of brain tissue

The results of H&E staining in **Figure 5A** revealed that the hippocampal neurons in the normal group exhibited abundant numbers and a well-organized arrangement, with most of them being round or oval-shaped. In contrast, the model group displayed a reduction in hippocampal neuron count, accompanied by disordered cellular arrangement and irregularly shaped nuclei exhibiting pyknosis and dark staining.

Nissl staining as **Figure 5B** showed that the Nissl bodies of normal group mice were abundant, neatly arranged, and darkly stained, most of which were triangular. The Nissl bodies of model group mice were reduced in number, disordered in arrangement, and shallow in color, with pyknosis and different shapes. Compared with the model group, the Nissl bodies of each treatment group increased in number and morphology. The statistical results of Nissl staining in **Figure 5E** showed that compared with the normal group, the number of Nissl bodies in the model group was significantly reduced ($P < 0.01$); Compared with the model group, the number of Nissl bodies in each treatment group was significantly increased ($P < 0.01$). These results suggest that the administration of LJC can enhance hippocampal nuclear condensation and augment the quantity of nissellites in the cerebral cortex.

3.6 The Effects of LCJ on the immune capacity of thymus and spleen

To evaluate the effect of LJC on immune organs, H&E staining of thymus and spleen and ACP and LDH activities of spleen were performed.

Results as depicted in **Figure 5C**, the thymic lobules of mice in the normal group exhibited clear boundaries between the medulla and cortex, with densely and regularly arranged abundant lymphocytes within the cortex. Conversely, in the model group, there was an indistinct boundary between the medulla and cortex, accompanied by an enlarged diffusion of medulla and a reduced number of sparsely arranged lymphocytes. Following LJC administration, structural damage to the thymus was alleviated in each group as evidenced by a relatively clear boundary between the medulla and cortex, a significant reduction in medullary area, and a relatively close arrangement of lymphocytes.

We can see from **Figure 5D** that the spleen structure of the normal group mice appeared histologically intact, with a distinct demarcation between the red pulp and white pulp, and an abundance of densely arranged lymphocytes within the white pulp. In contrast, mice in the model group exhibited compromised spleen structure characterized by an indistinct boundary between the red pulp and white pulp, a loose overall architecture, reduced area of white pulp, and decreased number of loosely arranged lymphocytes. However, following LJC treatment, there was evident amelioration in spleen structural damage across all groups as indicated by a clear demarcation between the red pulp and white pulp, increased area of white pulp, and enhanced lymphocyte population.

The results of ACP and LDH in spleen are shown in **Figure 5F-G**. Compared with the normal group, the activity of ACP and LDH in spleen of mice in the model group decreased, but the change of LDH activity was not statistically significant. Compared with the model group, LJC-H could significantly enhance the activities ($P < 0.05, 0.01$), suggesting that LJC could restore the pathological damage of spleen and thymus in immunocompromised mice with sleep disorders.

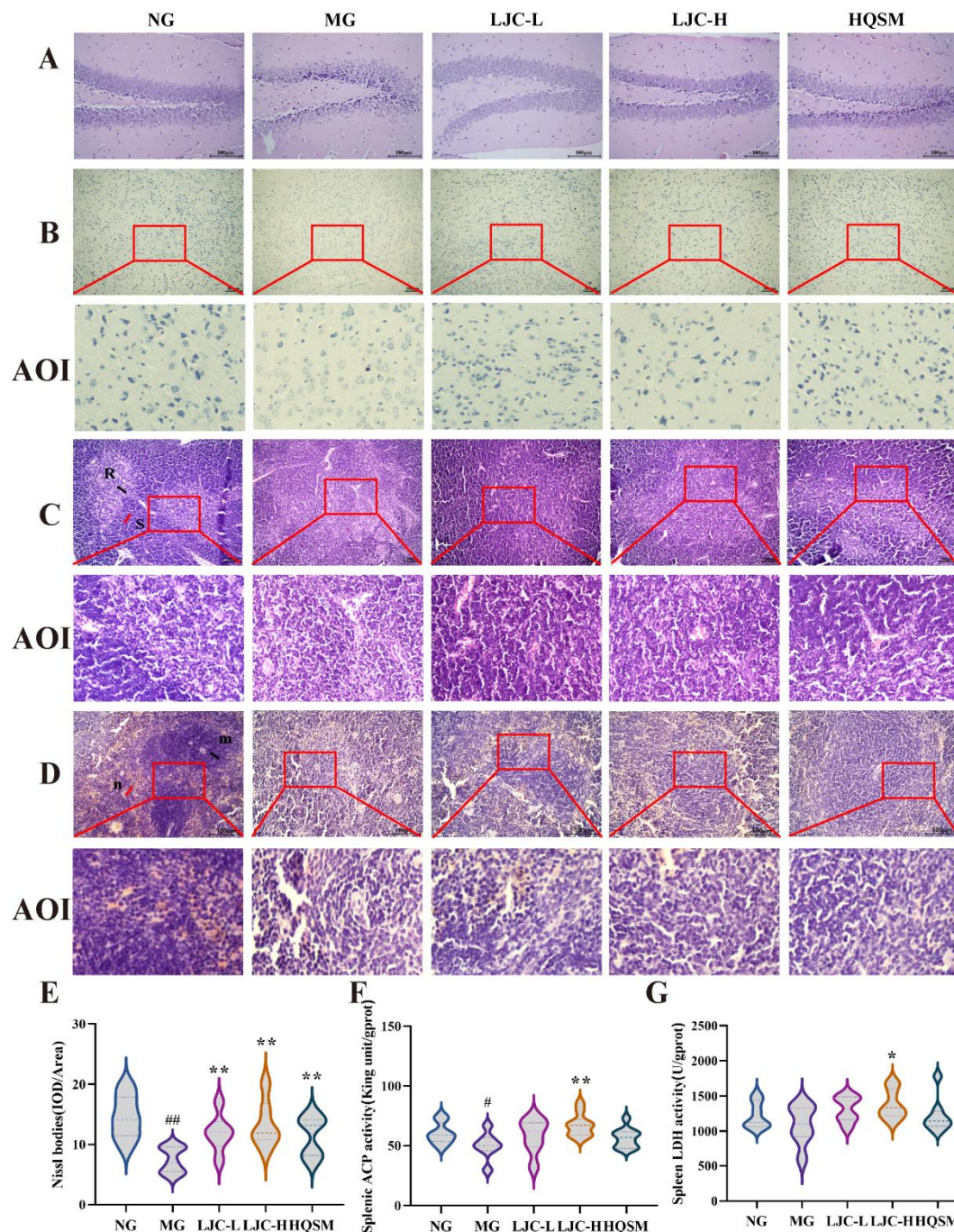


Figure 5. Histopathological evaluation of brain tissue, thymus and spleen. (A) H&E staining of hippocampus; (B) Nissant staining; (C) Thymus H&E staining (200×), R: thymus medulla, S: thymus cortex; (D) H&E staining of spleen (400×), m: white pulp area of spleen, n: red pulp area of spleen; (E) Nissl bodies; (F) Spleen ACP viability; (G) Activity of LDH in spleen. Data are expressed as the mean $\pm$ SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compare with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compare with the model group.

3.7 The effects of LJC on GABA and Glu contents and GABA pathway proteins in mice

The levels of GABA and Glu in the blood of the mice were determined by HPLC. Compared with the normal group, the serum Glu content and Glu/GABA ratio in the

model group were significantly increased ($P < 0.05$, 0.01); Compared with the model group, the serum Glu content and Glu/GABA ratio in the LJC group were significantly decreased ($P < 0.05$, 0.01). GABA content was just opposite. This suggests that LJC may affect the sleep state of sleep-deprived mice by regulating Glu and GABA levels (**Figure 6A-F**).

To evaluate the effect of LJC on GABA pathways, we examined the protein expression of GABRA1, GAT1, and GAD in brain tissue. As shown in **Figure 7A-E**, immunohistochemistry results showed that compared with the normal group, the expression of GABRA1, GAT1, and GAD protein in the model group was significantly decreased ($P < 0.01$). After LJC administration, all the indexes showed varying degrees of increase compared to the model group. Conversely, the expression of GABAT1 protein exhibited an opposite trend. As shown in **Table 2** and **Figure 7F**, the WB results showed that compared with the normal group, the protein expression of GABRA1, GAT1 and GAD in the model group was decreased, and the contents of these three proteins were increased to varying degrees after LJC administration.

Table 2 WB results of GABA pathway protein expression

Group	GABRA1	GAD	GAT1
NG	0.509±0.045	0.906±0.020	0.889±0.075
MG	0.352±0.075 [#]	0.761±0.051	0.824±0.082
LJC-L	0.503±0.191	0.889±0.015	0.843±0.031
LJC-H	0.658±0.189 [*]	0.992±0.024 [*]	0.923±0.105

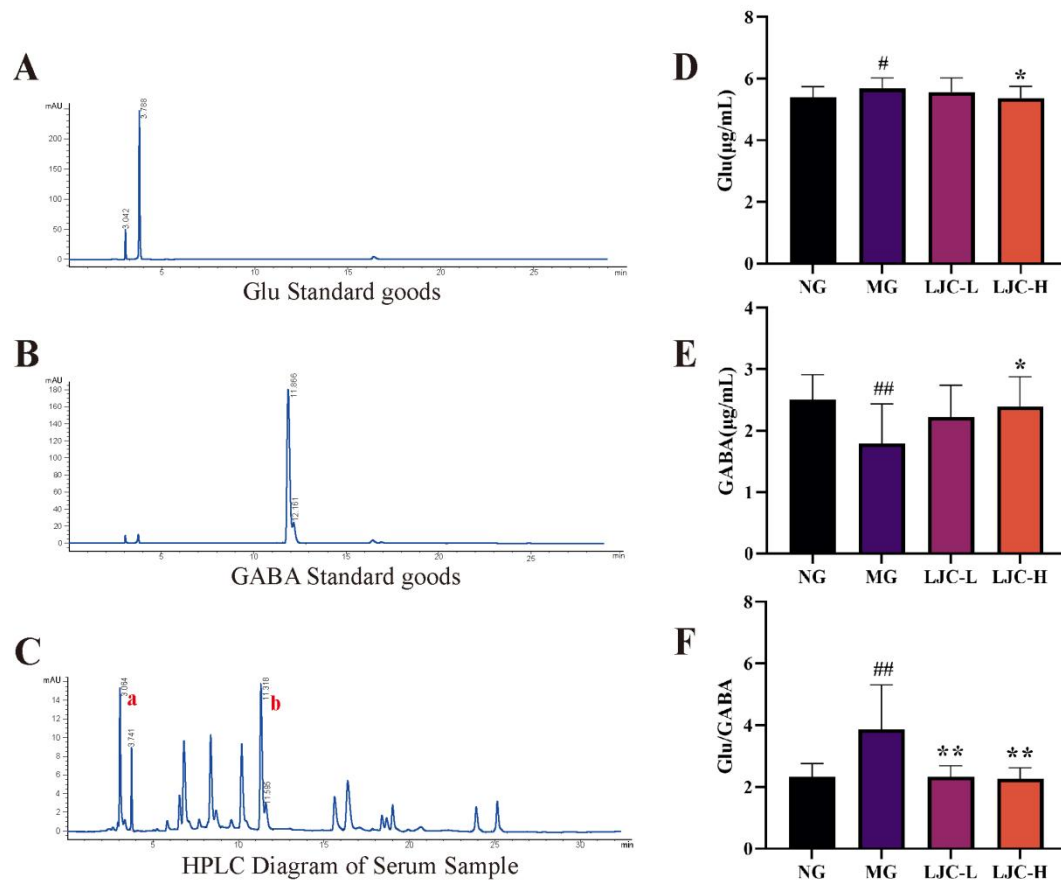


Figure.6. The effects of LJC on GABA and Glu contents. (A) HPLC diagram of Glu standard; (B) HPLC diagram of GABA standard; (C) HPLC representative map of serum samples; (D) Glu content; (E) GABA content; (F) Glu/GABA. Data are expressed as the mean $\pm$ SD. #P < 0.05, ##P < 0.01 compare with the control group; *P < 0.05 and **P < 0.01 compare with the model group.

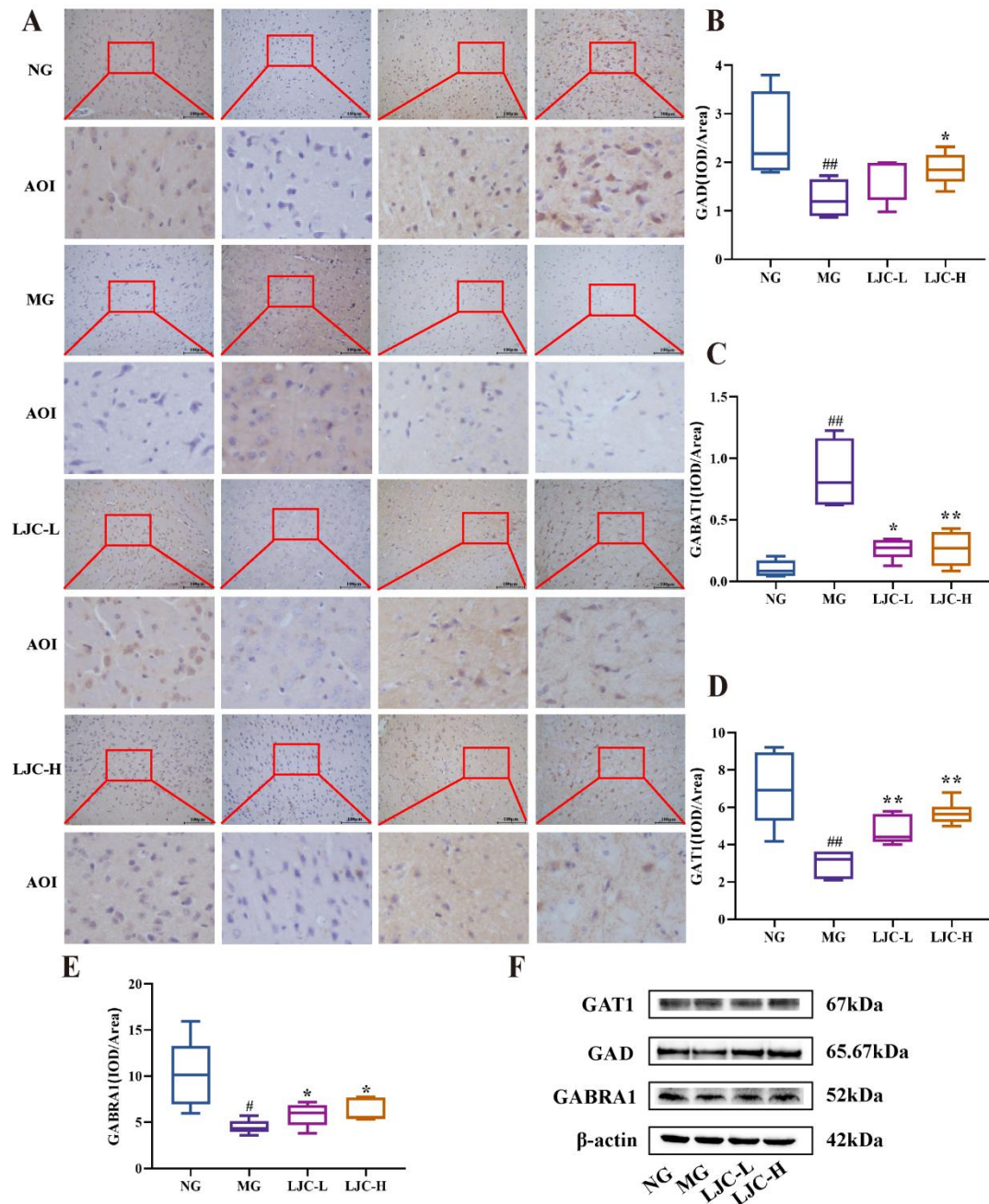
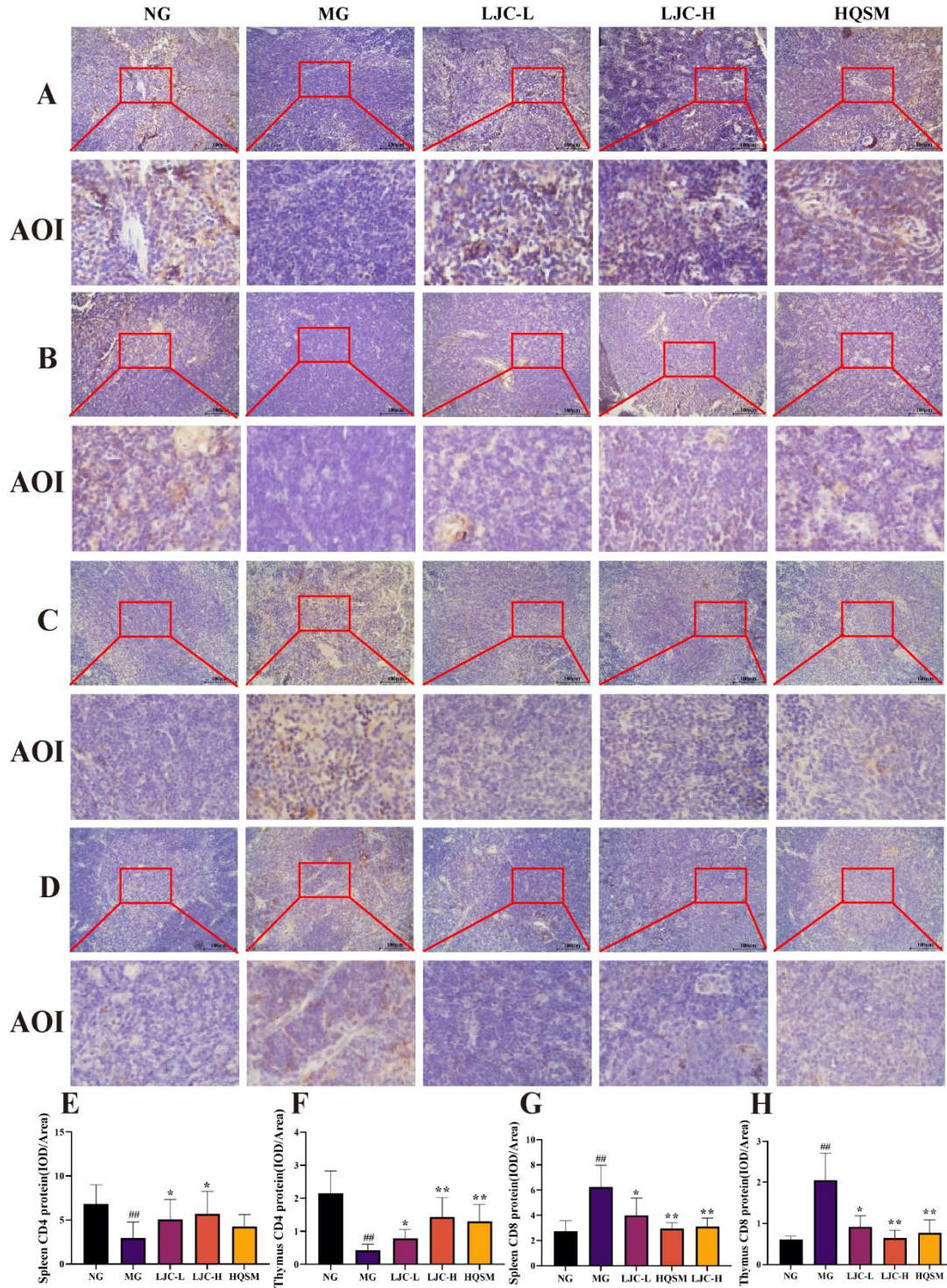


Figure 7. The effects of LJC on GABA pathway proteins in brain tissue secretion. (A) Immunohistochemical representation of GABA pathway proteins (400 $\times$); (B) Statistical diagram of GAD protein expression; (C) Statistical map of GABAT1 protein expression; (D) Statistical diagram of GAT1 protein expression; (E) Statistical diagram of GABRA1 protein expression; (F) Representative diagrams of GABA pathway protein expression. Data are expressed as the mean $\pm$ SD. # P < 0.05, ## P < 0.01 compare with the control group; * P < 0.05 and ** P < 0.01 compare with the model group.

3.8 The expression of CD4 $^{+}$ and CD8 $^{+}$ proteins

The CD4 $^{+}$ content of the spleen and thymus in the model group showed a significant decrease compared to the normal group, as depicted in **Figure 8** (P < 0.01). Conversely, administration of LJC significantly increased CD4 $^{+}$ cell count when

589 compared to the model group ($P < 0.05, 0.01$). However, there was an opposite trend
 590 observed for CD8+ content.



591
 592 **Figure.8. The expression of CD4+ and CD8+ proteins.** (A) Representative diagram of splenic CD4+
 593 immunohistochemistry (400×); (B) thymic CD4+ immunohistochemical representative plot (400×); (C) CD8+
 594 immunohistochemical representative diagram of spleen (400×); (D) thymic CD8+ immunohistochemical
 595 representation (40×); (E) Statistical graph of splenic CD4+ expression; (F) Statistical diagram of thymic CD4+
 596 expression; (G) Statistical diagram of splenic CD8+ expression; (H) Statistical plot of thymic CD8+ expression.

Data are expressed as the mean $\pm$ SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compare with control group; $^*P < 0.05$ and $^{**}P < 0.01$ compare with model group.

4. Discussion

The immune system's primary function is to monitor and interpret external incursions and potential threats, subsequently initiating appropriate defensive responses. Simultaneously, it also monitors the internal organ status, enhances resistance against disturbances, and maintains homeostasis. Disruption of the body's immune equilibrium can impact sleep quality ^[61]. The act of sleeping is an essential physiological function. With the acceleration of the pace of life and the mounting social pressure, an increasing number of individuals are afflicted by sleep disorders ^[62]. The presence of sleep disorders is commonly observed in various diseases and is believed to disrupt the physiological processes that regulate the immune system, thereby contributing to the development of diseases ^[25,63,64]. Our experiment simulated the bad living habits of people in today's society, such as sleep disorders, poor diet, and overwork, and the results showed that the mice exhibited a phenomenon of "consumption" and displayed symptoms consistent with "deficiency syndrome", which could be effectively ameliorated by LJC administration.

Autonomic activity experiment, elevated plus maze test and open-field test are commonly used to evaluate the sedative, anxiolytic or stimulating effects of drugs on model mice ^[65]. Compared with the model group, LJC significantly reduced the motor activity of sleep-deprived mice, prolonged the duration of sleep induced by pentobarbital (50 mg/kg), and had a good sedative and restaging effect.

A growing body of research is revealing that the seemingly autonomous immune and nervous systems mutually monitor, interpret, and regulate each other. The nervous system actively monitors, interprets, and regulates the behavior of immune cells and their intricate functions, while neurons and glial cells in the nervous system are subject to immune surveillance, with their physiological function even relying on factors derived from immune cells ^[61]. The neuro-immune units (NIU) serve as the fundamental structural entities for neuro-immune interaction within tissues and organs ^[66]. NIU refers to the co-localization of neuronal processes and immune cells in

specific anatomical regions of the body, as well as the association of neuropeptides, neurotransmitters, cytokines, and other effector molecules for information transmission, ultimately forming a bidirectional functional interaction unit. They are distributed across various tissues including bone marrow, thymus, spleen, lung, skin, intestine, and brain. The nervous system directly or indirectly interacts with immune cells through neurotransmitters and neuroregulatory factors. Immune cells perceive changes in local tissue environment's neurotransmitter levels via autocrine or paracrine signaling mechanisms to regulate the body's immune response [67-72].

The hippocampus is the most vulnerable of the brain regions under the influence of stress or other pathological conditions [73]. Previous studies have shown that the effects of sleep deprivation on the hippocampus are deterministic [74,75]. Recently, Zhao [76] et al. used APTw imaging to detect disordered hippocampal protein suppression in sleep-deprived rats, and hippocampal APTw signals were positively correlated with the number of surviving neurons on Nissl staining. In this study, it was observed that the hippocampus of model mice exhibited damage characterized by reduced and disorganized neuronal population as well as a decrease in the number of nissl bodies. LJC demonstrated significant efficacy in ameliorating hippocampal damage and promoting an increase in nissl body count.

GABA/Glu is a pair of inhibitory/excitatory neurotransmitters that act on neurons and are widely present in the central nervous system, involved in sleep regulation [77]. Patients with sleep disorders often have decreased GABA levels, which may be accompanied by decreased GAD, GATs, and GABRA1 levels, and increased GABAT levels [78,79]. The results showed that LJC could up-regulate the serum GABA content, down-regulate the ratio of Glu content to Glu/GABA, increase the expression of GABRA1, GAT1 and GAD, and decrease the expression of GABAT1 in the model mice. Thus, sleep disorders can be improved.

Studies have found that sleep deprivation (both chronic and acute) can lead to changes in the function of the immune system, including changes in organs such as the thymus, and the levels of cells and factors such as lymphocytes, neutrophils, natural killer cells, and IL-6 [13,22,80,81]. As the main lymphoid organ, the thymus is an

important site for the development, differentiation, and maturation of T lymphocytes [82]. The spleen is the largest secondary lymphoid organ in the body, where a large number of T and B lymphocytes settle down and participate in a wide range of immune functions [83,84]. Wu [85] et al. found that TCM was able to increase the activities of ACP and LDH in the spleen of immunocompromised mice. Acid phosphatase (ACP) and lactate dehydrogenase (LDH) are important markers of macrophage activation [86,87]. The pathological changes and related indicators of thymus and spleen were analyzed. The results showed that the structure of thymus and spleen in the model group was damaged, and the activities of spleen ACP and LDH were decreased. LJC could significantly improve the damage of thymus and spleen, and up-regulate the decrease of spleen ACP and LDH activities.

B cell and T cell activation can be used as a general indicator of specific immune activation [88]. When stimulated by antigen, B lymphocytes transform into plasma cells and secrete antibodies to perform their functions [89-91]. The value of hemolysin can reflect the ability of B cell proliferation and differentiation [92-94]. Our experimental results showed that the levels of serum hemolysin, serum immunoglobulin IgA, IgG, and IgM in the model group were significantly decreased, and LJC could significantly up-regulate the values and enhance the immune function.

As the center of the immune system, the levels of CD4⁺ and CD8⁺T lymphocytes can reflect the immune situation of the human body. When the ratio of CD4⁺ and CD8⁺T lymphocytes decreases, it indicates that the cellular immune-related functions are in a certain inhibition [95,96]. The results of this study showed that compared with the model group, LJC could significantly increase CD4⁺ content, decrease CD8⁺ content, and increase CD4⁺/CD8⁺ ratio. Delayed-type hypersensitivity (DTH) response is an immune response mediated by CD4 T cells, which can reflect the cellular immune function of mice by measuring the degree of foot swelling after 24 hours [97]. The paw swelling degree of the model group was significantly reduced, while that of the LJC group was significantly increased.

In addition, the complement system is an important component of the non-specific immune system [98,99]. Complement mediators, particularly C3a are able

to activate neutrophils, mast cells, monocytes/macrophages, T cells, B cells and etc [100]. The activation of the complement system occurs through three main pathways-classical, lectin, and alternative-that converge in C3 activation. And C4 is involved in the classical complement pathway and the lectin complement pathway [101]. Previous studies have shown that the levels of serum complement C3 and C4 in immunocompromised people are lower than those in healthy people [102,103]. Similar results were obtained in the present experiment. The levels of complement C3 and C4 in the serum of the model mice were significantly decreased, and those in each dose group of LJC were significantly increased. However, the number of white blood cells and lymphocytes in the blood decreased significantly, and the number of central granulocytes increased significantly. LJC administration significantly increased the number of white blood cells and lymphocytes, and decreased the number of neutrophils.

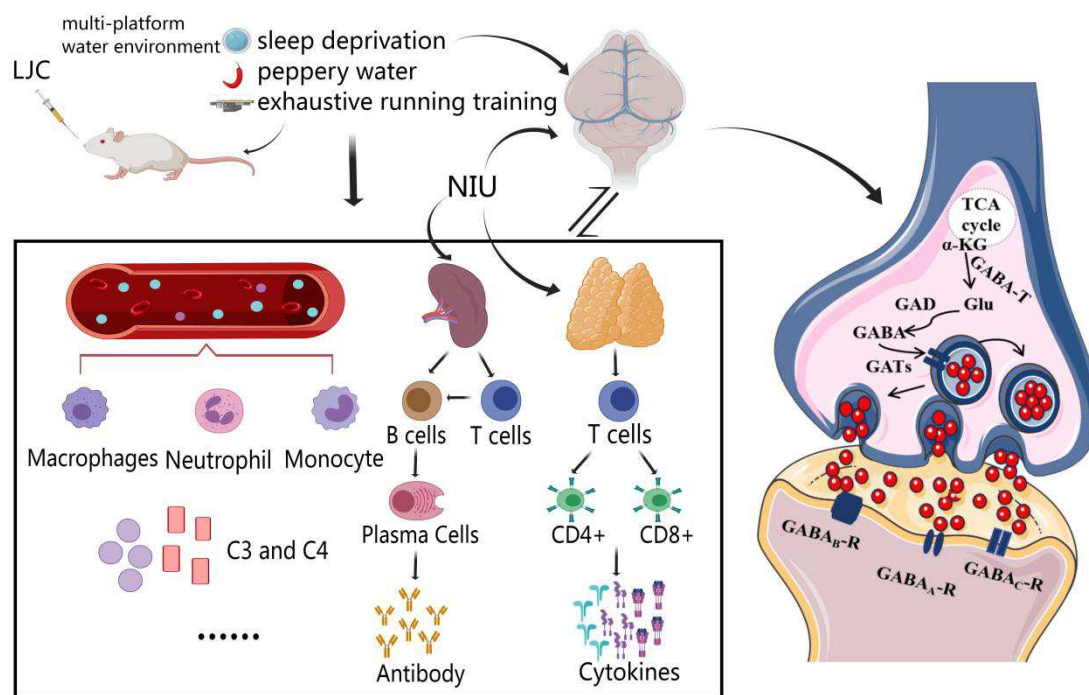


Figure.9. Schematic representation of the mechanism underlying the ameliorative effects of LJC on immunocompromised and sleep-deprived mice through modulation of the GABAergic neuro-immune system.

5. Conclusion

This study found that LJC can regulate the content of GABA/Glu and the

expression level of GABA metabolic pathway-related proteins in the brain of mice, enhance the specific and non-specific immune function of model mice, and thus improve the state of low immunity and sleep disorders in model mice. Therefore, it is possible to consider developing LJC as a new drug or health food to cope with the increasing number of sub-healthy people with low immunity and sleep problems.

Abbreviations

LJC, Modified Qiyuan Paste;
HQSM, Huangqi Shengmai Drink;
GABA, γ -aminobutyric acid;
Glu, Glutamate;
GAD, Glutamic acid decarboxylase;
GABAT, γ -aminobutyric acid transaminase;
CATs, GABA transporters;
GABRA1, GABAA receptor $\alpha 1$ subunit;
EPM, Elevated plus maze;
OFT, Open - field test;
DTH, Delayed hypersensitivity;
EDTA, Ethylene diamine tetraacetic acid;
ELISA, Enzyme-linked immunosorbent assay;
RIPA, Radioimmunoprecipitation;
PVDF, Polyvinylidene fluoride;

Ethics approval and consent to participate

All animal experiments were approved by ethics committee of Zhejiang University of Technology (20221125Abzz0100999262).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author's Contribution

MR, JJJ and MQL designed and performed research, analyzed data, and wrote the paper. XLSH provided article writing guidance. ZYX, NW and ZHZ analyzed data. YJD, WFX and JHH modified article. BL guided experiment and revised article. NHJ provided project guidance. GYL conceptualized the project. SHC provided funding acquisition.

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Not applicable.

Availability of data and materials

All data associated with this study are present in the paper. Any information for this study is available by contacting the corresponding authors upon reasonable request.

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