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Research Article
Metabolomics Study of Insomnia and Intervention Effects of Wuweiningshen Decoction on PCPA Induced Insomnia Rats by UPLC/Q-TOF-MS

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Abstract

Background and Objective: Insomnia is a common sleep disorder and lots of people suffered from it. Wuweiningshen decoction (WWNSD), a clinical experience prescription of Traditional Chinese Medicine, was widely used with very good curative effect. This study aimed to discover the unclear mechanism of WWNSD on insomnia. **Materials and Methods:** It was used to study the effects of WWNSD on insomnia through the brain tissue histopathology experiment and the pharmacodynamic experiments of the prolonged sleep time test (PSTT). The metabolomics method by UPLC/Q-TOF-MS was adopted to explain the biochemical mechanisms of the sedative-hypnotic effects. The key points were verified in the metabolic pathways by enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (RT-PCR). **Results:** Combined with the results of the brain tissue histopathology experiment and the insomnia pharmacodynamic tests, it was demonstrated that WWNSD could relieve the symptoms of insomnia. Sixteen metabolites were found in the metabolomics study. It was determined that WWNSD could effectively regulate the amount of small molecule metabolism on insomnia, including tricarboxylic acid cycle, tryptophan metabolism, bile acid metabolism and purine metabolism. The expression of serotonin with ELISA and MT1 and MT2 with RT-PCR proved what we discovered. **Conclusion:** This experiment provided a multi-pathway comprehensive regulation mechanism of WWNSD and established a base for searching and building innovative, high potential and fewer side effects drugs in insomnia treatment. It was also a powerful foundation for the application and scientific explanation of clinical prescription.

Key words: Metabolomics, wuweiningshen decoction, traditional Chinese medicine, insomnia, multi-pathway, serotonin, UPLC/Q-TOF-MS

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Insomnia is a kind of sleep problem which are characterized by persistent difficulties to fall asleep or maintain sleep[1]. Insufficient sleep with poor quality results from daytime dysfunction and contributes to irritability, depression and anxiety[2]. More and more people suffered from it. The primary types of drugs for treating insomnia are prescribed including benzodiazepines, non-benzodiazepines and antidepressants. The first two kinds of medicine exhibit sedative-hypnotic effects by binding to GABA receptors, contributing to the potentiation of the GABAergic function[3]. Antidepressants, due to their antagonism with serotonin and norepinephrine, are also used to treat insomnia[4]. Nevertheless, all of these drugs reflect different levels of anxiety, sleep disorders and pharmacological dependence, especially in long time users. The frequent use of these drugs even brings about the concomitant increased risks of mortality and morbidity[5]. As a result, there are an increasing number of insomniacs that are looking for alternatives because of the significant adverse reactions[6].

Traditional Chinese medicine (TCM) has been used in the treatment of insomnia for more than 2000 years in China[1]. Meanwhile, it has been focused on Western countries, like America, for the past few years[7]. Wuweiningshen decoction (WWNSD), an experiential prescription for treating insomnia, was set up by famous Chinese medicine professor Lide Zhang based on the Wuweizi formula. Many insomniacs were cured or effectively alleviated symptoms. This prescription consists of six Chinese medicine, namely *Schisandra chinensis* (Wuweizi, SC), *Ziziphi spinosae* (Suanzaoren, ZP), *Polygala* (Yuanzhi, PG) *Acanthopanax* (Ciwujia, AP), *longan aril* (Longyanrou, LA) and *Poria* (Fuling, P) those are all medicinal and edible plants, most frequently used TCM in treatment of insomnia[1,8].

Metabolomics is a powerful method to systemically analyze small-molecule endogenous metabolites in organs or cells, applied to monitor the dynamic alternation of metabolites induced by both internal and external factors[9]. As a key to systems biology, it can provide new methods to research the mechanism of TCM and accelerate to reveal of the interaction between TCM and biological response system. The integration of chromatography, mass spectrometry and other modern analytical techniques, made up of the metabonomics technology platform, has expedited the development of metabolomics[10]. In particular, the ultra-high performance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC/Q-TOF-MS) technique has many advantages in metabonomic studies, such as more sensitive detection, shorter analytical time and better reproducibility[11].

In this study, the brain tissue histopathology experiment and the pharmacodynamic experiments were used to study the effects of WWNSD on insomnia. Then the metabolomics method by UPLC/Q-TOF-MS was adopted to explain the biochemical mechanisms of the sedative-hypnotic effects. In analyzing the changes in serum metabolites, the metabolic pathways related to WWNSD were discussed, which would be useful for anti-insomnia medicine.

MATERIALS AND METHODS

Study area: This study was carried out from March, 2019 to April, 2020 at Liaoning University of Traditional Chinese Medicine.

Reagents and materials: The MS grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). The MS grade formic acid was obtained from Fisher Scientific (MA, USA). Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA). The 4-chloro-DL-phenylalanine (PCPA) was purchased from Sigma (Company Inc, USA). Barbitone sodium and pentobarbitone sodium were obtained from JK Chemical Company (Beijing, China). The WWNSD which contained SC, ZP, PG AP, LA and P was purchased from the Affiliated Hospital of Liaoning University of Traditional Chinese Medicine (Shenyang, China) and identified by Professor Yanjun Zhai (School of Pharmacy, Liaoning University of Traditional Chinese Medicine).

Preparation of WWNSD: The WWNSD was prepared by SC, ZP, PG, AP, LA and P, which was reflux extracted from 50% ethanol twice. The reflux extracts were combined and concentrated on 0.1875 g mL^{-1} (for the pharmacodynamic experiments) and 0.3038 g mL^{-1} (for the metabolomics study).

Ethics statement: All animals were handled with human care throughout the experiment and all treatments were strictly by the Use of Medicine Ethics Review Committee for animal experiments of Liaoning University of Traditional Chinese Medicine.

Animals: Specific pathogen-free 60 male mice (18-22 g) and 48 male rats (230-270 g), were prepared (the experimental animal centre of Liaoning Changsheng Biological Technology Company). They were kept under controlled conditions (22°C, RH 50-60%) with food and water supplied randomly.

Animal preparation and sample processing: In the pharmacodynamic experiments, 60 mice used in PSTT were divided into 4 groups randomly, including control group, low dose 1 group (L_1 group) (0.0625 g mL^{-1}), middle dose 1 group (M_1 group) (0.125 g mL^{-1}) and high dose 1 group (H_1 group) (0.1875 g mL^{-1}), 0.2 mL once a day. Except for saline given to the control groups, the other groups received oral WWNSD continuously for 30 days.

In the metabolomics test, 48 rats were assigned to 6 groups. The control group was given saline. Three groups received oral WWNSD (0.0338, 0.1012 and 0.3038 g mL^{-1}) and the model group and the positive group were administered saline and diazepam (DZ, 0.045 mg mL^{-1}) separately, 2 mL once a day, continuously for 14 days, followed by i.p., PCPA (0.3 g kg^{-1}) for 4 days at day 5. After a day of last given WWNSD, all groups were anaesthetized and sacrificed. Blood was collected from the abdominal aorta. Serum was separated from centrifugation at 3000 r min^{-1} for 10 min and frozen in liquid nitrogen at -80°C. Part of the brain was anatomized, washed with saline and stained with formalin for histopathological observation. Other brain tissues were rapidly removed and frozen in liquid nitrogen for ELISA and RT-PCR.

Histopathology analysis: The brain samples of PCPA-induced insomnia rats were fixed with 10% formalin and embedded in paraffin blocks. Brain tissue block sections were installed on slides, deparaffinized in xylene, dehydrated in alcohol and it was prepared in sections with a thickness of 5 μm. Hematoxylin and eosin were used to stain the sections and observed under light microscopy.

Preparation of metabolomic samples: The defrosted serum samples of PCPA-induced insomnia rats were prepared before analysis. A 200 μL serum sample was added to 600 μL methanol-acetonitrile (50:50, v/v) and stored at -20°C. The mixture was transferred into Captiva filtration plates and the supernatant was blow-dried with nitrogen, redissolved with 50 μL methanol-acetonitrile (50:50, v/v), then vortex for 15 sec. After a while, the supernatant was moved to the auto-sampler vials.

UPLC/Q-TOF-MS conditions: Chromatography was performed on Agilent 1290 series UPLC system (Agilent Technologies, Inc., USA). The separation was conducted on a 4.6×100 mm Poroshell SB-C 18 column (Agilent Technologies, Inc., USA). The column temperature was maintained at 40°C. Ultrapure water with 0.01% formic acid (A) and methanol-acetonitrile (50:50, v/v) (B) constituted the mobile phase and the gradient elution programs in positive mode as follows: 5-100% B for 0-30 min, 100-100% B for 30-37 min and in negative mode as follows: 5-100% B for 0-30 min. The flow rate was 0.6 mL min^{-1} and the sample injection volume was 5 μL.

The Q-TOF-MS analysis was performed on an Agilent-6550 Q-TOF mass spectrometry with a mass range of m/z 50-1000. The optimum parameters of the Mass detector were set as follows: Capillary voltage, 4000 V in positive mode and -3500 V in negative mode, drying gas temperature, 280°C, drying gas flow rate 13 L min^{-1}, nebulizer pressure, 50 psig, sheath gas temp, 300°C, sheath gas flow 12 L min^{-1}, fragmentation voltage, 150 V, OCT 1 RF Vpp, 750 V.

Multivariate analysis: The molecular feathers extraction (MFE) algorithm in The MassHunter Workstation software (version B.06.00 qualitative analysis, Agilent) was used to extract the compound information from the original data and extracted compound list of each file was exported as compound exchange format (.cef) files for further mass profiler professional (14.9, Agilent) statistical analysis. Then, chromatographic peak matching was performed with Agilent MPP 12.0 software and one-way ANOVA multiple test and PCA analysis were used in the data to screen biomarkers with VIP >1, $p<0.05$, folder change >2.

Enzyme-linked immunosorbent assay: The levels of serotonin in rats' brain samples from control, model, DZ and low, middle, high dose groups were detected by enzyme-linked immunosorbent assay (ELISA) on a basis of the instructions of the manufacturer (Shanghai Lianshuo Biotechnology Co., LTD, Shang Hai, China, No. 201910202004).

Molecular data: Each rat's brain tissue sample including control, model, DZ and three doses of WWNSD groups was used for RNA extraction with TRIZOL (Invitrogen, Carlsbad, CA, USA). The cDNA was produced by gDNA removal reaction and reverse transcription reaction with PrimeScript® RT Reagent Kit with gDNA Eraser (Takara Biomedical Technology (Beijing) Co., Ltd.). Differential gene expression was evaluated by qPCR, with SYBR® Premix Ex Taq™II (Tli RNase H Plus), ROX plus kit (Takara Biomedical Technology (Beijing) Co., Ltd.). The expression ratios of MT1 and MT2 were quantified against the housekeeping gene β-actin. The following primers (5'-3' end) were used: For MT1, forward primer (AGATCTCGGAATGGACCCCA) and reverse primer (AGGAGCAGCAGCTCTTCTTG) for MT2, forward primer (GCAGCGATCTCTCGTTGATCT) and reverse primer (AGGAGCAGGATCCATCTGTGG), for β-actin, forward primer (CGCGAGTACAACCTTCTTGC) and reverse primer (CGTCATCCATGGCGAACTGG).

Statistical analysis: All quantitative data were expressed as the Mean±Standard deviation as indicated. The differences were analyzed by One-way Analysis of Variance (ANOVA) using SPSS software, version 19.0.

RESULTS

Insomnia pharmacodynamic experimental results of PSTT: For 60 mice (15 per group), it was performed by injecting with pentobarbital sodium (60 mg kg^{-1}). With the disappearance of righting reflex, it was observed the mice extended sleep time. Compared with the control group, it was found that the mice extending sleep time in the H1 group were lengthened ($p<0.05$) (Table 1, Fig. 1).

Results of histopathological examination: The brain samples of PCPA-induced insomnia rats were used in histopathological observation. Compared with the control group, the granular layer of nerve cells of the model group of the hippocampus was arranged disorderly, edematous and with pale cytoplasm, confirming the change of PCPA in the nerve cells in the hippocampus of rats.

And from the histopathological observation, it was shown that the result of the high dose group was similar to the DZ group. Three different doses were effective against the morphological structure of nerve cells in contrast to the model group (Fig. 2a-f). Therefore, it was observed that three doses of WWNSD had the effect of treating insomnia and the high dose was better.

Results of metabolomic profiles: Typical total ion chromatograms (TIC) of PCPA-induced insomnia rats' serum samples were presented in Fig. 3a-f and 4a-f using the optimal reversed-phase UPLC/Q-TOF-MS conditions described above. To identify the diversities and connections between the groups, this study used principal components analysis. It was carried out by the quality analyzer 12.0 of MPP. From the three-dimensional plots (Fig. 5a, b), samples in the same group were clustered together, but the model group was significant to distinguish from the others. This result suggested the serum metabolite compositions of the model group had differences from the other groups. The position of the WWNSD groups was near to the control group, indicating there was a tendency to back onto normal after treatment.

The different endogenous metabolites in the serum of PCPA-induced insomnia rats were presumed by using the ID Browsers Mentlin (Agilent), which provided the information about small molecule metabolites with significant differences (t-test, $p<0.05$). The speculated molecular formula was searched for KEGG, HMDB and other databases to identify the possible chemical constitutions. According to this study, 16 different metabolites were identified (Table 2, 3), involving tricarboxylic acid cycle, tryptophan metabolism, bile acid metabolism and purine metabolism. The connections between these identified compounds were demonstrated in Fig. 6.

Table 1: Results were in PSTT

Groups	Sleeping time (min)
Control group	41.56±8.58
L_1	45.54±10.01
M_1	47.38±12.67
H_1	50.47±11.05*

Compared with the control group, *p<0.05

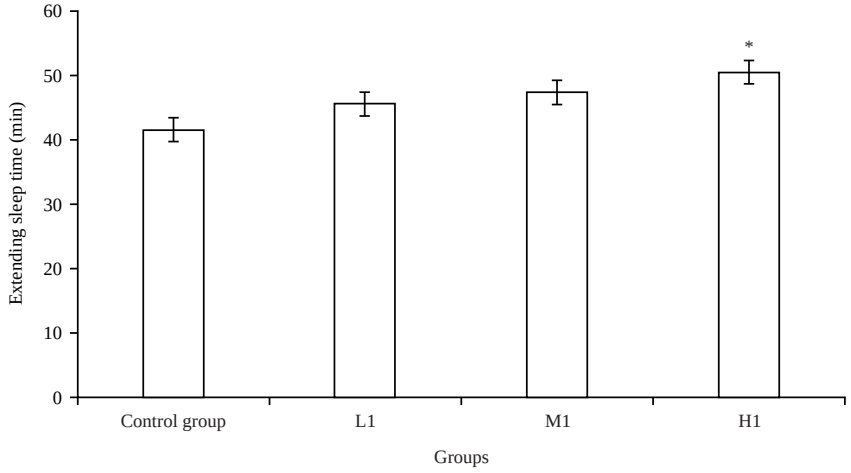

Fig. 1: Results were in PSTT

Data were expressed as Mean±Standard deviation, *p<0.05 compared with the control group

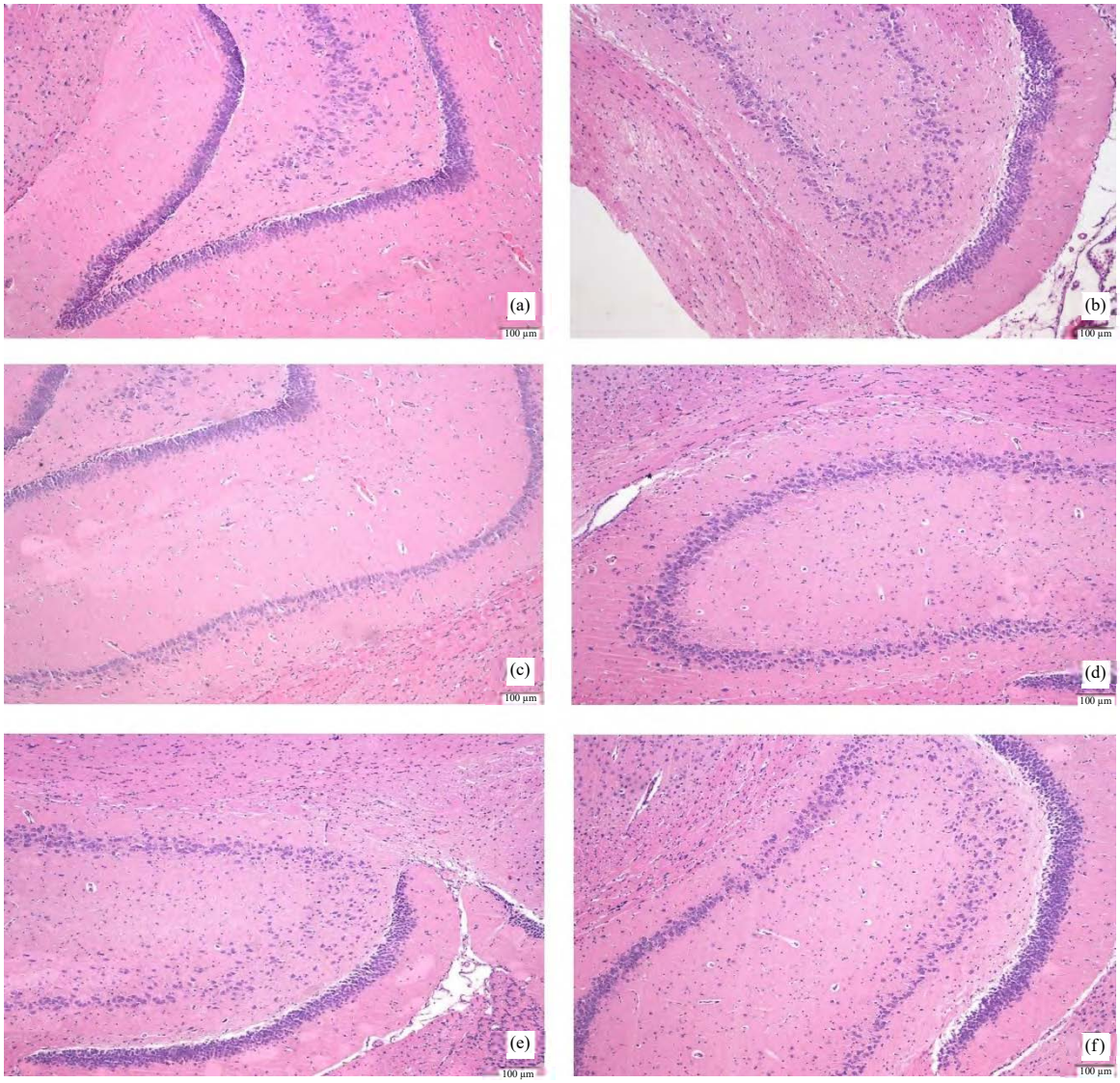

Fig. 2(a-f): Histopathological findings in hippocampus stained with HE, (a) HE-stained section of control group 10×10, (b) HE-stained section of PCPA induced insomnia group 10×10, (c) HE-stained section of DZ group 10×10, (d) HE-stained section of high dose WWNSD group 10×10, (e) HE-stained section of middle dose WWNSD group 10×10 and (f) HE-stained section of low dose WWNSD group 10×10

Table 2: Identification results of different metabolites by UPLC/Q-TOF-MS in positive mode

RT (min)	m/z	Molecular formula	Metabolites	Regulation	Metabolic pathway
1.75	303.2328	$C_{20}H_{30}O_2$	Retinyl ester	Up	Retinol metabolism
6.26	119.0261	$C_4H_6O_4$	Succinic acid	Up	Tricarboxylic acid cycle
8.19	349.0549	$C_{10}H_{13}N_4O_8P$	Inosinic acid	Down	Purine metabolism
8.21	285.2258	$C_{10}H_{12}N_4O_6$	Xanthosine	Down	Purine metabolism
8.22	122.0265	$C_3H_7NO_2S$	L-Cysteine	Down	Bile acid metabolism
8.29	170.0112	$C_3H_7NO_5S$	Cysteic acid	Down	Bile acid metabolism
17.30	248.0322	$C_8H_{10}NO_6P$	Pyridoxal 5'-phosphate	Up	Tryptophan metabolism
25.06	409.2955	$C_{24}H_{40}O_5$	Cholic acid	Up	Bile acid metabolism
30.60	466.3169	$C_{26}H_{43}NO_6$	Glycocholic acid	Up	Bile acid metabolism
30.89	500.3048	$C_{26}H_{45}NO_6S$	Taurodeoxycholic acid	Up	Bile acid metabolism

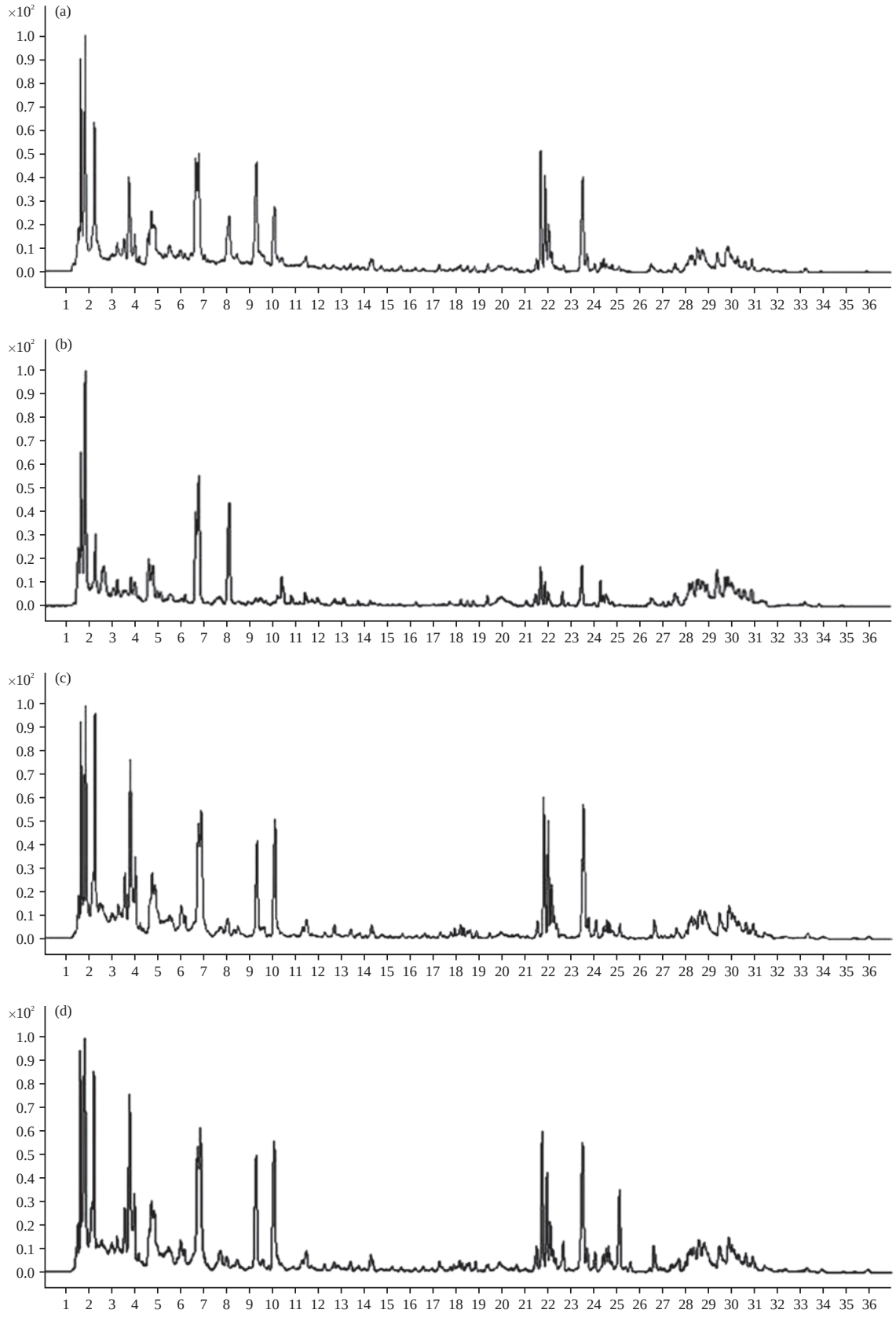

Fig. 3(a-f): Continue

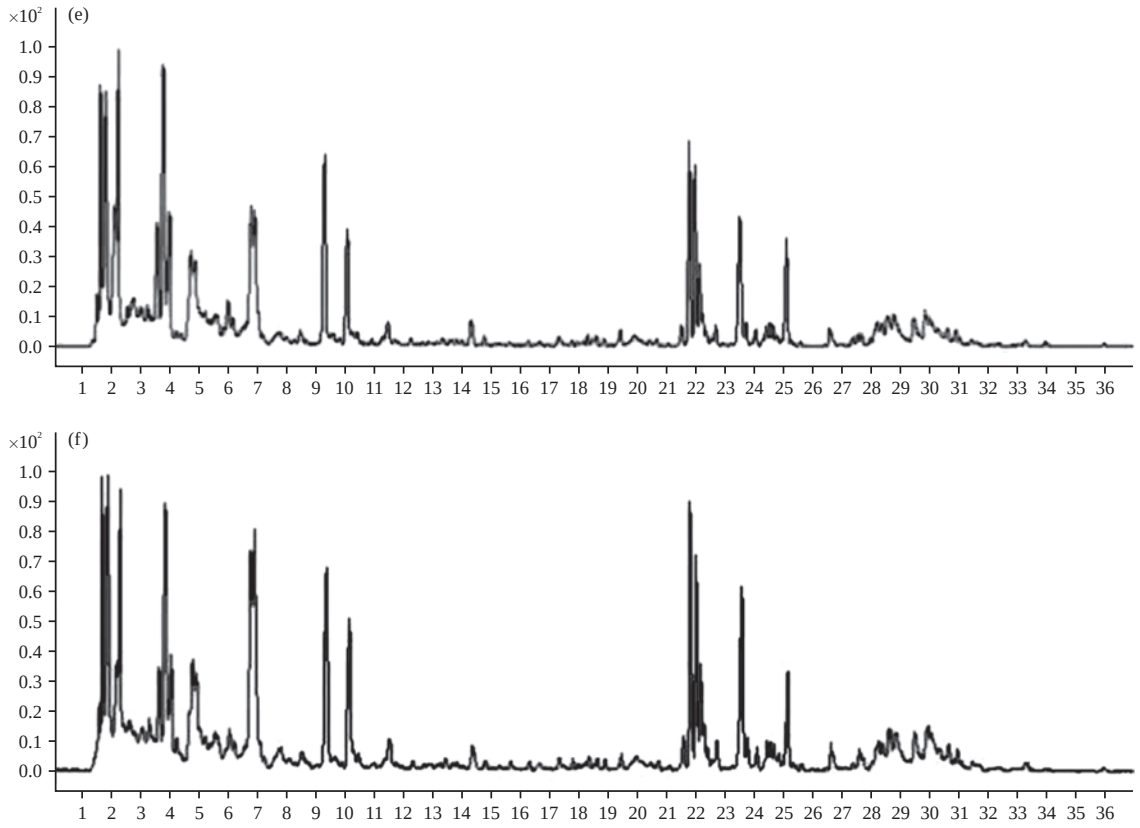

Fig. 3(a-f): TIC chromatograms derived from UPLC/Q-TOF-MS in positive mode, (a) Control group, (b) Model group, (c) DZ group, (d) Low dose group, (e) Middle dose group and (f) High dose group

Table 3: Identification results of different metabolites by UPLC/Q-TOF-MS in negative mode

RT (min)	m/z	Molecular formula	Metabolites	Regulation	Metabolic pathway
1.73	250.0951	$C_{10}H_{13}N_5O_3$	Deoxyadenosine	Up	Purine metabolism
8.32	203.0821	$C_{11}H_{12}N_2O_2$	L-Tryptophan	Up	Tryptophan metabolism
10.71	247.1094	$C_{13}H_{16}N_2O_3$	6-Hydroxymelatonin	Up	Tryptophan metabolism
14.67	228.0276	$C_5H_{12}NO_7P$	5-Phosphoribosylamine	Down	Purine metabolism
15.07	204.0658	$C_{11}H_{11}NO_3$	5-Methoxyindole acetate	Up	Tryptophan metabolism
24.78	391.2861	$C_{24}H_{40}O_4$	Deoxycholic acid	Down	Bile acid metabolism

Table 4: Results of ELISA and RT-PCR

Group	MT1	MT2	Serotonin (pg mL^{-1})
Control group	1.002±0.064	1.004±0.092	100.10±4.13
Model group	0.290±0.020**	0.509±0.038**	45.27±1.42**
DZ group	0.901±0.062**##	0.780±0.071**##	81.20±3.73##
Low dose group	0.644±0.053**##	0.573±0.068**	53.55±1.55**##
Middle dose group	0.645±0.042**##	0.682±0.059**##	65.44±1.93**##
High dose group	0.807±0.062**##	0.810±0.062**##	59.42±1.46**##

Compared with the control group, **p<0.01 and compared with the model group, ##p<0.01

Determination of the level of serotonin: Serotonin was determined by the ELISA technique and the results were shown in Table 4, Fig. 7. The content of serotonin was decreased significantly in the model group (p<0.01), compared with the control group. After the treatment of WWNSD, the contents of serotonin in low, middle and high groups were shown a rising trend significantly in comparison with the model group (p<0.01), indicating that WWNSD could up-regulate the level of serotonin to improve insomnia.

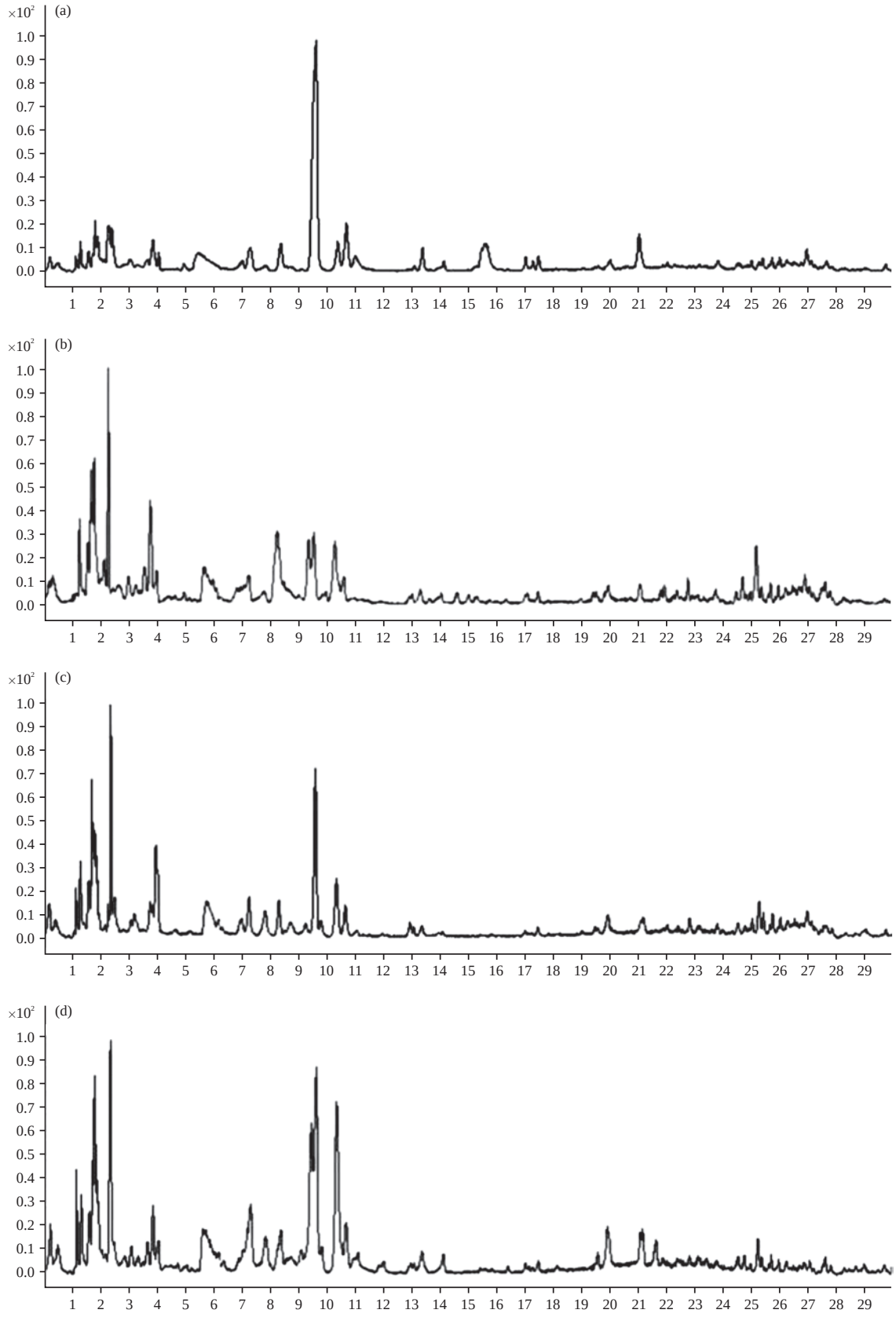

Fig. 4(a-f): Continue

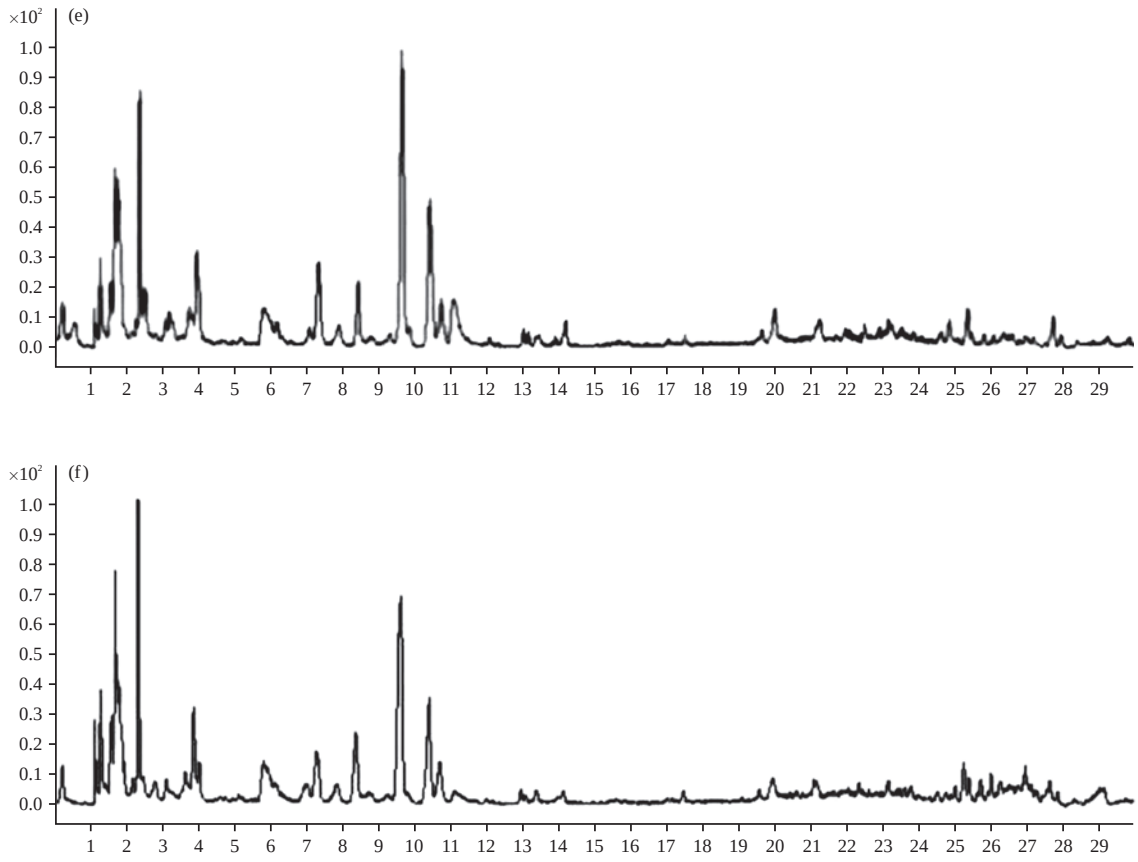

Fig. 4(a-f): TIC chromatograms derived from UPLC/Q-TOF-MS in negative mode, (a) Control group, (b) Model group, (c) DZ group, (d) Low dose group, (e) Middle dose group and (f) High dose group

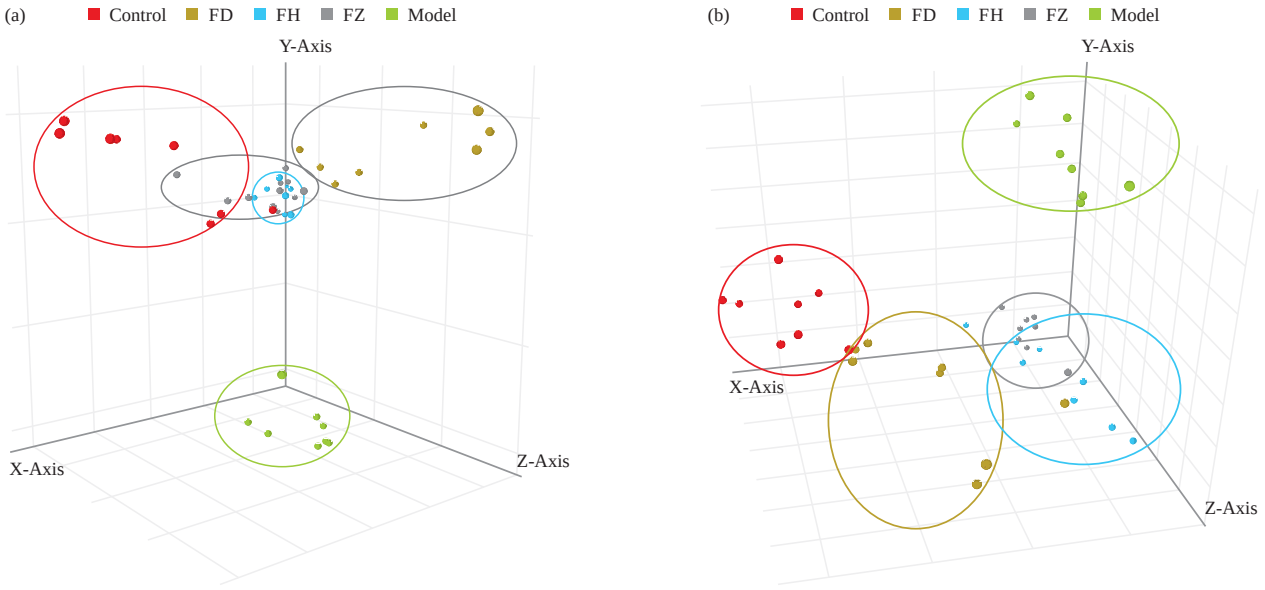

Fig. 5(a-b): Principle component analysis of each group, (a) Positive mode and (b) Negative mode

Each colour point represented a sample. The first, second and third principal components were displayed on the X, Y and Z-axis, respectively. These three components represented the largest fraction of the overall variability. Red: Control group, Yellow: Low dose group, Blue: High dose group, Gray: Middle dose group, Green: Model group

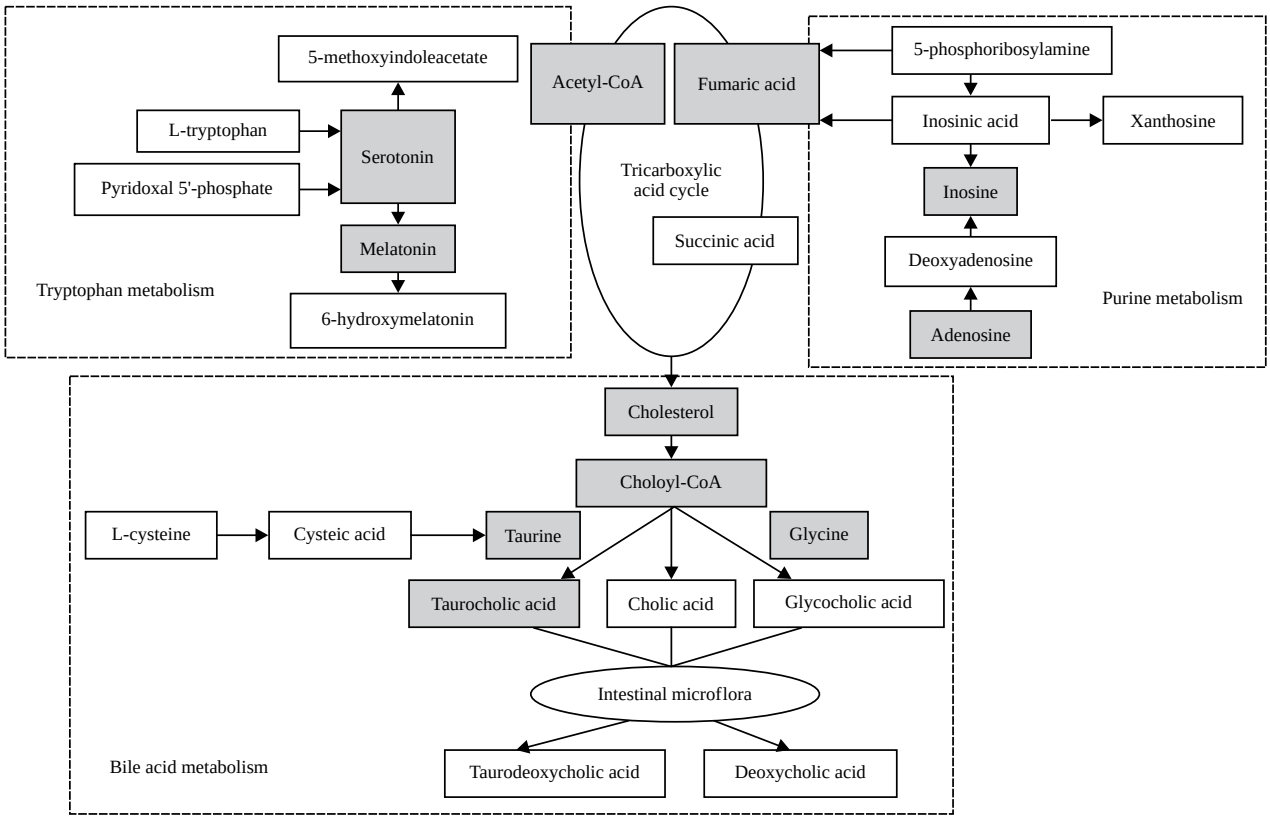

Fig. 6: Metabolites and pathways related to the potential mechanism of WWNSD curing insomnia
Metabolites with dark dashed areas represent important connections

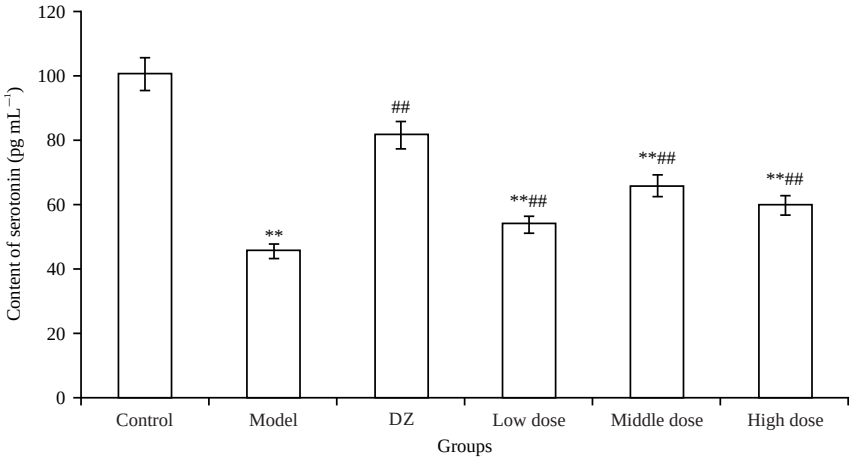

Fig. 7: Contents of serotonin in each group
Data were expressed as Mean±Standard deviation, **p<0.01 compared with the control group and ##p<0.01 compared with the model group

Determination of mRNA levels: The molecular data were acquired to confirm the metabolites found in further steps by the RT-PCR technique. It was used to determine the two mRNAs, containing MT1 and MT2, associated with melatonin which was the key point in the tryptophan metabolism pathway and it was illustrated that the results were in Fig. 8. The mRNA expressions of MT1 and MT2 notably declined in the model group (p<0.01). The expression levels of MT1 and MT2 were up-regulated after giving WWNSD (p<0.01), especially in the high dose group.

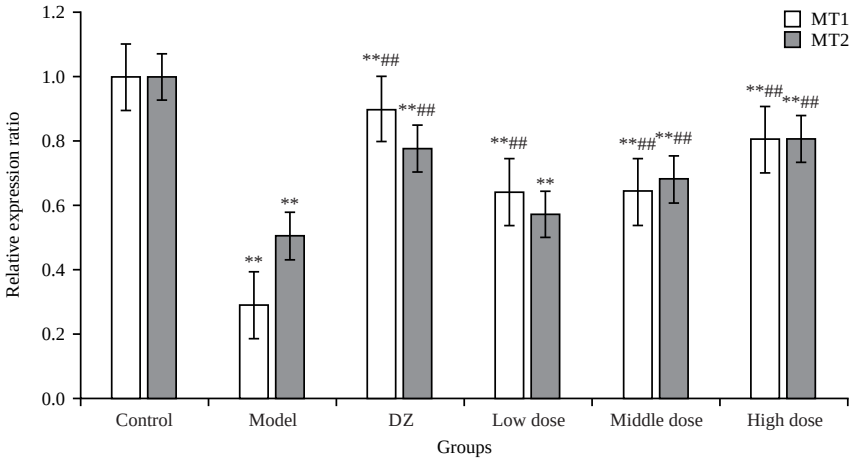

Fig. 8: Expression levels of mRNAs

Data were expressed as Mean±Standard deviation, **p<0.01 compared with the control group and ##p<0.01 compared with the model group

DISCUSSION

In the study, based on PCPA induced insomnia rats, sixteen dominating components of WWNSD including retinyl ester, succinic acid, inosinic acid, xanthosine, L-cysteine, cysteic acid, pyridoxal 5'-phosphate, cholic acid, glycocholic acid, taurodeoxycholic acid, deoxyadenosine, L-tryptophan, 6-hydroxymelatonin, 5-phosphoribosylamine, 5-methoxyindole acetate, deoxycholic acid, which illustrated the material basis of WWNSD improving sleep. Furthermore, the mechanism of action was speculated as follows.

The tricarboxylic acid cycle (TCA cycle) played an important role to allow the release of energy from carbohydrates, fat and amino acids[12]. Succinic acid was intermediate in the TCA cycle. In this study, it was shown that the decreased levels of succinic acid slowed down energy metabolism in the aggravated disease which caused direct damage to the nerve system through the accumulation of pernicious metabolites in the body[13]. Compared with the model group, the increasing contents of succinic acid in WWNSD treated groups indicated that WWNSD could accelerate the TCA cycle, owing to removing neurotoxins and improving sleep.

Several metabolic pathways are involved in the TCA cycle, including tryptophan metabolism. This pathway was made up of some key compounds, including tryptophan, pyridoxal 5'-phosphate, serotonin, melatonin, 6-hydroxymelatonin and 5-methoxyindole acetate. Tryptophan, an essential amino acid was the precursor of both serotonin and melatonin. Serotonin was known as a neurotransmitter in the brain, a clotting factor in platelet and a neurohormone in organs throughout the body and modulated sleep and higher contents of serotonin could help to improve sleep[14-17]. Pyridoxal 5'-phosphate was the active form of vitamin B6 serving as a coenzyme for the synthesis of serotonin[18]. Melatonin was produced by the pineal gland in animals, which regulated circadian rhythms. A high level of melatonin was considered to promote sleep[19-21]. 5-methoxyindole acetate was synthesized from serotonin under the action of the relevant enzymes and its level could indirectly reflect the synthesis and metabolism of serotonin[22]. The 6-hydroxymelatonin was an important melatonin metabolite, converted via the action of cytochrome P450s in the endoplasmic reticulum by the variation of melatonin[23]. In the present study, after administrating WWNSD, the levels of tryptophan, pyridoxal 5'-phosphate, 6-hydroxymelatonin and 5-methoxyindole acetate were increased, compared with the model group. It was speculated that WWNSD could improve sleep quality by impacting the key metabolites in tryptophan metabolism, which were capable of improving sleep.

Bile acids played an important role in metabolic homeostasis by facilitating excretion, absorption and fat transport[24]. Bile acid metabolism was triggered by cholesterol. Acetyl-CoA was the beginning of the TCA cycle and served as a precursor of cholesterol. Firstly, cholesterol was converted into choloyl-CoA through a series of reactions, which then were used in three reactions. The three derivatives from these three reactions were taurocholic acid, cholic acid and glycocholic acid, which left the cells to interact with intestinal microflora and became taurodeoxycholic acid and deoxycholic acid[25]. Moreover, cysteine and cysteic acid were converted into taurine and participated in this reaction. Higher taurine could cause alteration of neurotransmitter homeostasis and lead to neurological disorders[26]. We found cysteine and cysteic acid were lower compared with the model group, suggesting

the restoring function of WWNSD to the unbalance of the nervous system with dysfunction of bile acid metabolism[27]. There were decreases in serum concentrations of many bile acids in the model group, including cholic acid, glycocholic acid and deoxycholic acid, which might be connected with high blood viscosity which resulted in neurodegenerative disease[28]. The intervention of WWNSD could raise them, indicating that WWNSD could influence the function of bile acid biosynthesis to treat insomnia.

In purine metabolism, adenosine, one of the most important purines, consisted of DNA and RNA. Meanwhile, Adenosine was considered not only to protect nervous system function but also to make sure a central nervous system with a correct development and function[29]. Deoxyadenosine was a derivative of the nucleoside adenosine. It could act as two kinds of toxins at sufficiently high levels. One was an immunotoxin, limiting the function or exterminating immune cells and the other was an endogenous metabolite causing unhealthy effects at high levels for a long time[30]. Xanthosine was an intermediate in purine metabolism. Previous studies had shown neurologic disorder-induced high levels of adenosine, inosine and xanthosine[31]. Results indicated that there was a tendency towards normal regulation in adenosine deoxyadenosine, xanthosine and inosinic acid after administration of WWNSD, which could act on immune cells to reduce neurotoxicity.

CONCLUSION

Through metabonomics combined with ELISA and RT-PCR technology, this study illustrated the mechanism of WWNSD on PCPA induced insomnia rats by regulating tricarboxylic acid cycle, tryptophan metabolism, bile acid metabolism and purine metabolism, which was basic evidence to search and exploit innovative, high potential and fewer side effects drugs in insomnia treatment.

SIGNIFICANCE STATEMENT

Based on clarifying the WWNSD's efficacy, this study further explores the mechanism of WWNSD in the treatment of insomnia. It can be indicated that WWNSD treats insomnia by regulating multiple biomarkers and metabolic pathways. This study is the first to illustrate the mechanism of WWNSD in treating insomnia by regulating the tricarboxylic acid cycle, tryptophan metabolism, bile acid metabolism and purine metabolism, providing better insights on WWNSD and contributing to its clinical application.

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