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RESEARCH ARTICLE

Relationship of nocturnal concentrations of melatonin, gamma-aminobutyric acid and total antioxidants in peripheral blood with insomnia after stroke: study protocol for a prospective non-randomized controlled trial

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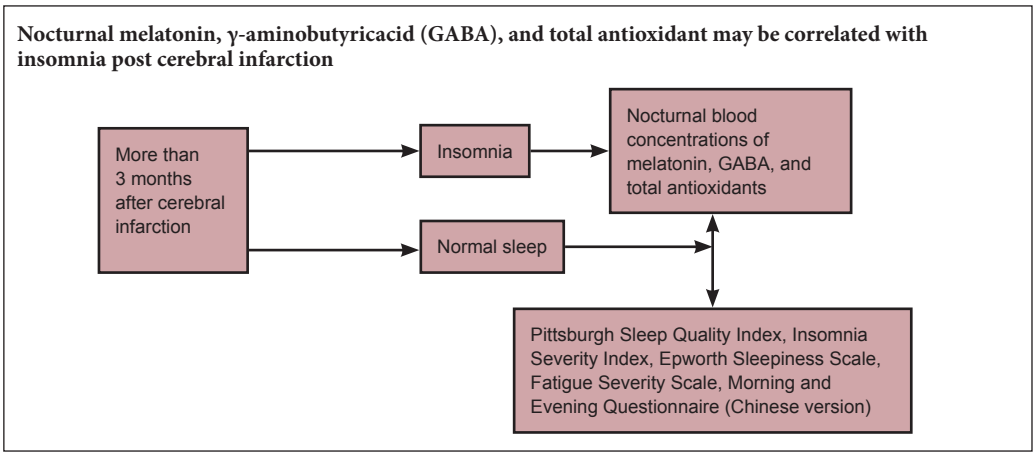
Graphical Abstract



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Abstract

Melatonin and gamma-aminobutyric acid (GABA) have been shown to regulate sleep. The nocturnal concentrations of melatonin, GABA and total antioxidants may relate to insomnia in stroke patients. In this prospective single-center non-randomized controlled clinical trial performed in the China Rehabilitation Research Center, we analyzed the relationship of nocturnal concentrations of melatonin, GABA and total antioxidants with insomnia after stroke. Patients during rehabilitation of stroke were recruited and assigned to the insomnia group or non-insomnia group. Simultaneously, persons without stroke or insomnia served as normal controls. Each group contained 25 cases. The primary outcome was nocturnal concentrations of melatonin, GABA and total antioxidants in peripheral blood. The secondary outcomes were Pittsburgh Sleep Quality Index, Insomnia Severity Index, Epworth Sleepiness Scale, Fatigue Severity Scale, Morningness-Eveningness Questionnaire (Chinese version), and National Institute of Health Stroke Scale. The relationship of nocturnal concentrations of melatonin, GABA and total antioxidants with insomnia after stroke was analyzed and showed that they were lower in the insomnia group than in the non-insomnia group. The severity of stroke was higher in the insomnia group than in the non-insomnia group. Correlation analysis demonstrated that the nocturnal concentrations of melatonin and GABA were associated with insomnia after stroke. This trial was registered at ClinicalTrials.gov, identifier: NCT03202121.

Key Words: *nerve regeneration; stroke; insomnia; melatonin; γ-aminobutyric acid; total antioxidants; sleep-related scales; National Institute of Health Stroke Scale; neural regeneration*

Introduction

Sleep disturbance, especially insomnia, is a common complication after ischemic stroke for patients during the rehabilitation of cerebral infarction (Leppävuori et al., 2002; Suh et al., 2014; Kim et al., 2015). More than half of ischemic stroke patients have insomnia complaints (Leppävuori et al., 2002) and poor quality of sleep may greatly impede stroke rehabilitation and induce other complications. Thus, it is of importance to

study causes of insomnia in post-stroke patients, especially during rehabilitation of cerebral infarction.

Melatonin is a pineal hormone with a peak nocturnal secretion (Claustrat et al., 2005). Melatonin typically acts in coordination with circadian rhythms to regulate sleep function (Hajak et al., 1996; Rodenbeck et al., 1999; Micic et al., 2015). The peak of melatonin secretion is around 12:00 midnight to 3:00 a.m. (Claustrat et al., 2005; Atanassova et al., 2009). Along with other antioxidants, melatonin also functions as an effective neuroprotective enzyme against neurodegeneration and ischemic brain injury (Wang, 2009; Shekleton et al., 2010; Andrabi et al., 2015; Milanlioglu et al., 2016). Thus, melatonin has an important role in acute ischemic stroke, where its rhythm is impaired and it undergoes nocturnal decrease (Fiorina et al., 1996; Beloosesky et al., 2002; Atanassova et al., 2009; Ritzenthaler et al., 2009). Gamma-aminobutyric acid (GABA) is likewise a strong sleep regulator that may activate GABA receptors as well as inhibitors of waking processes (Gottesmann, 2002; Harrison, 2007). It is known that GABA levels in humans are strongly associated with the impairment of patients after acute ischemic stroke (Paik and Yang, 2014; Blicher et al., 2015). Antioxidants are important for the balance of oxidation by scavenging free radicals and are important markers of insomnia in post-stroke patients. However, to our knowledge, there has been no report of the simultaneous measurement of levels of melatonin, GABA and antioxidants in the blood of patients during convalescence from ischemic stroke or their association with insomnia complications in post-stroke patients.

Therefore, this prospective single-center randomized controlled clinical trial was designed to investigate the relationship between the nocturnal concentrations of melatonin, γ-aminobutyric acid and total antioxidants with insomnia after stroke by comparing levels in stroke patients with or without insomnia and normal controls.

Design and Methods

Study design

This is a prospective single-center non-randomized controlled clinical trial in the China Rehabilitation Research Center. Stroke patients during rehabilitation have been recruited since July in 2014. After screening according to inclusion and exclusion criteria, patients were assigned to either the insomnia or non-insomnia group. Simultaneously, persons without stroke or insomnia served as normal controls. Each group contained 25 cases. The primary outcome was nocturnal concentrations of melatonin, γ-aminobutyric acid and total antioxidants in peripheral blood. The secondary outcomes were Pittsburgh Sleep Quality Index, Insomnia Severity Index, Epworth Sleepiness Scale, Fatigue Severity Scale, Morningness-Eveningness Questionnaire (Chinese version), and National Institute of Health Stroke Scale. The relationship of nocturnal concentrations of melatonin, γ-aminobutyric acid and total antioxidants with insomnia after stroke was analyzed (**Figure 1**).

This trial was designed in January 2012. Patients have been recruited since July 2014, and patient recruitment will be finished in December 2017. Data analysis will be finished in December 2018.

Study participants

We screened stroke patients who had treatment at the China Rehabilitation Research Center from January 2012 to June 2014 and were in a period of rehabilitation based on electronic medical records.

(1) Stroke patients with sleep disorders:

Inclusion criteria: patients presenting with all of the following criteria were considered for study inclusion:

- Infarction occurred in the middle cerebral artery blood supply area (identified by medical record, magnetic resonance imaging, magnetic resonance angiography, computed tomography or computed tomography angiogram)
- Diagnostic criteria for insomnia according to the Diagnostic and Statistical Manual of Mental Disorders (4th edition) (American Psychiatric Association, 1994)
- Course of disease ≥ 3 months
- Mini-Mental State Examination (Pangman et al., 2000) > 27
- Age range from 50 to 70 years old
- Right handedness

Exclusion criteria: Patients with one or more of the following conditions were excluded from this study:

- Cognitive and language disorders
- History of rheumatism, cancer, severe liver and kidney dysfunction, benign prostatic hyperplasia, severe cardiac insufficiency
- High-risk sleep apnea, *i.e.*, STOP-Bang Questionnaire scores (Nagappa et al., 2015) ≥ 3
- Unexplained limb pain, getting up in the night many times to urinate or restless legs syndrome
- Frequency of application of sleeping drugs > once/week or the use of psychotropic drugs, such as anti-anxiety and depression drugs, and antipsychotic drugs
- Frequency of drinking coffee and other stimulating drinks > three times/week
- Drug or alcohol abuse
- Insomnia caused by poor sleeping conditions, such as noise, light, and bedmate interference
- Insomnia before stroke
- Hamilton Depression Scale scores (Hamilton, 1960) > 20 or Hamilton Anxiety Scale scores (Hamilton, 1959) > 14
- Participation in other clinical trials

(2) Stroke patients with normal sleep:

Patients did not have insomnia symptoms, *i.e.*, not in accordance with the Diagnostic criteria for insomnia of the Diagnostic and Statistical Manual of Mental Disorders (4th edition) (American Psychiatric Association, 1994). The remaining screening criteria were the same as those with stroke sleep disorders.

Control group: Patients who had treatment at the China Rehabilitation Research Center from January 2012 to June 2014

were used as controls because they did not suffer from stroke or insomnia.

Inclusion criteria: patients presenting with all of the following criteria were considered for study inclusion

- Mini-Mental State Examination (Pangman et al., 2000) > 27
- Age range from 50 to 70 years old
- Right handedness

Exclusion criteria: patients with one or more of the following conditions were excluded from this study:

- Stroke patients
- Diagnostic criteria for insomnia according to the Diagnostic and Statistical Manual of Mental Disorders (4^{th} edition) (American Psychiatric Association, 1994)
- Cognitive and language disorders
- High-risk sleep apnea, *i.e.*, STOP-Bang Questionnaire scores (Nagappa et al., 2015) ≥ 3
- Unexplained limb pain, getting up many times in the night to urinate or restless legs syndrome
- Frequency of application of sleeping pill > once/week or the use of psychotropic drugs, such as anti-anxiety and depression drugs, and antipsychotic drugs
- Frequency of drinking coffee and other stimulating drinks > three times/week
- Drug or alcohol abuse
- Hamilton Depression Scale scores (Hamilton, 1960) > 20 or Hamilton Anxiety Scale scores (Hamilton, 1959) > 14
- Participation in other clinical trials

Recruitment

We screened stroke patients who had treatment at the China Rehabilitation Research Center from January 2012 to June 2014 and were in a period of rehabilitation based on electronic medical records. We contacted patients directly for the purpose of the trial and recruited patients to participate in the trial. After providing informed consent, these potential participants were screened using the inclusion and exclusion criteria.

Sample size and allocation

Nocturnal concentrations of melatonin in persons aged 60 years old was 40 pg/mL on average (Zhao et al., 2003). Concentrations of melatonin were 12 pg/mL and 35 pg/mL in cerebral infarction persons with and without sleeping disorders. Standard deviation was estimated at 30. Taking $\beta = 0.1$ and power = 90% with a significance level of $\alpha = 0.05$, a final sample size of $n = 30$ per group was calculated using PASS 11.0 software (PASS, UT, USA). After screening according to the inclusion and exclusion criteria, 25 patients per group were included in the trial. Simultaneously, 25 normal controls were included. The sample size results were analyzed according to the intention-to-treat principle.

Blinding

Patients and physicians were not blinded to group information because insomnia involved subjective judgment and required medical diagnosis. The assessors were blinded to group information.

Outcome measures

Primary outcome measure

Nocturnal concentrations of melatonin, GABA and total antioxidants in peripheral blood

Two weeks before blood collection, patients avoided the use of alcohol, drugs, sleeping pills or antipsychotic drugs. Three days before measurement, patients were admitted to standard sleep laboratories to acclimatize to the environment. Blood samples from the ulnar vein were collected at 3:00 a.m. Patients were required to wear an eye mask before blood taking, and the laboratory remained in dim light to avoid affecting the secretion of melatonin. The collected blood was centrifuged immediately, and stored at −80°C.

High-performance liquid chromatography-mass spectrometry (HPLC-MS) was used to determine melatonin and GABA levels. (1) Standard concentration curves of melatonin and GABA. (2) Detection of melatonin using HPLC-MS: melatonin concentrations in the blood samples were measured with an ACQUITY UPLC H-Class system (Waters Corporation, Milford, MA, USA), which was connected to a Waters TQ-S mass spectrometer (Waters Corporation). A reversed-phased C18 BEH column (100 mm × 2.1 mm, 1.7 μm; Waters Corporation) was installed and the column temperature was maintained at 25°C. The flow rate was controlled at 0.35 mL/min. Acetonitrile was used as mobile phase A and 0.1% ammonium hydroxide aqueous solution was used as mobile phase B. A gradient elution was used with an initial ratio of mobile phase A:B = 30:70; at 1.5 minutes, adjusting the ratio to A:B = 90:10; and at 2.1 minutes, adjusting the ratio back to A:B = 90:10. Prior to the operation, the mobile phase was saturated with nitrogen to remove bubbles and dissolved air. The MS system (Waters Corporation) was running in the MRM mode. Ionization condition settings were desolvation at 400°C, source block at 150°C, cone voltage of 20 V, and capillary voltage of 2.7 kV. Eight melatonin standard solutions were tested to cover the range of melatonin concentrations in the blood samples. (3) Detection of GABA using HPLC-MS: GABA concentrations in the blood samples were separated on an ACQUITY UPLC H-Class system, and identified and quantified with a Waters TQ-S mass spectrometer. A Kinetex HILIC column (100 × 4.60 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) was installed, and the column temperature was controlled at 40°C. The flow rate was maintained at 1.0 mL/min. A gradient elution model was used with acetonitrile as mobile phase A and aqueous solution containing 10 mM ammonium acetate + 0.1% formic acid as mobile phase B. The initial ratio of mobile phase A:B was controlled at 80:20, and at 4 minutes, this ratio was changed to A:B = 65:35. The mobile phase was saturated with nitrogen to remove the air bubbles and dissolved air. The MS system was running in MRM cation scanning mode. The parameters of ion sources were as follows: CUR: 25.00, IS: 5,000.0, TEM: 600.00, GS1: 55.00, GS2: 60.00, CAD: High. Six GABA dilution series were tested against a standard curve to calibrate GABA levels by weighted least-

square regression mode.

Determination of total antioxidant concentrations using colorimetry: antioxidant levels were assessed with a colorimetric Antioxidant Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). Trolox Standards were prepared for obtaining a standard curve. ABTS substrate working solution was prepared by adding 25 mL of 3% hydrogen peroxide solution to 10 mL of ABTS substrate solution and vortexed. The solution was used within 30 minutes. The assays were prepared in a 96-well plate. For the Trolox standard curve, 10 mL of a Trolox Standard was added, followed by 20 mL of Myoglobin working solution. For the test samples, 10 mL of test sample was added, followed by 20 mL of Myoglobin working solution. Afterwards, 150 mL of ABTS substrate working solution was added to each well. After the plate was incubated for 5 minutes at room temperature, 100 mL of stop solution was added to each well. The endpoint absorbance values at 405 nm were measured using a plate reader. The plate was read within one hour. The assays were performed in duplicate and the average was used to determine the final value.

Secondary outcome measures

• Sleep-related scales included the Pittsburgh Sleep Quality Index, Insomnia Severity Index, Epworth Sleepiness Scale, Fatigue Severity Scale, and Morningness-Eveningness Questionnaire (Chinese version).

Pittsburgh Sleep Quality Index: an effective instrument used to measure the quality and patterns of sleep in adults established by Buysse et al. (1989). The measure consists of 19 individual items. Overall scores range from 0 to 21, where lower scores denote a healthier sleep quality.

Insomnia Severity Index: a brief self-reporting instrument measuring the patient's perception of nocturnal and diurnal symptoms of insomnia. It comprises seven items. Each item contains five grades with a total score of 28. A high score represents severe insomnia (Morin et al., 2011).

Epworth Sleepiness Scale: a scale intended to measure daytime sleepiness by use of a very short questionnaire. It was introduced by Dr. Murray Johns of Epworth Hospital in Melbourne, Australia. It contains eight items. The full score is 24. A high score represents obvious sleepiness tendency. The score of a normal person is 7.6 (Johns, 1991).

Fatigue Severity Scale: a 9–item questionnaire with questions related to how fatigue interferes with certain activities that rates severity. The items are scored on a 7 point scale with 1 = strongly disagree and 7 = strongly agree. This scale was developed by Krupp et al. (1989) for the treatment of systemic lupus erythematosus and multiple sclerosis. A higher score indicates greater fatigue severity.

Morningness-Eveningness Questionnaire (Chinese version): a self-rating scale for assessing circadian rhythm. It contains 19 items. The total score is 16–86. The tendency of the circadian rhythm of 163 healthy subjects was assessed by Zhang et al. (2006) from Guangdong General Hospital of China using the Morningness-Eveningness Questionnaire (Chinese version). Zhang et al. recorded sleeping habits through 2-week sleep record table. The reliability and validity of the scale were tested and the demarcation point was sought. Nineteen items were identified as two factors: sleep phase factor and best performance time factor. Demarcation point in Chinese version: absolute morning type, 70–86; moderate morning type, 63–69; intermediate type, 50–62; moderate evening type, 43–49; absolute evening type, 16–42. The Cronbach coefficient (Chinese version) was 0.701–0.738; the Spearman-Brown split-half reliability was 0.584–0.697; and for Retest reliability the data reached an acceptable level of psychometrics. The Morningness-Eveningness Questionnaire (Chinese version) has good psychometric characteristics. The new demarcation points can distinguish the morning and night types effectively.

• National Institute of Health Stroke Scale: a tool used to quantify stroke severity. The full score is 42. A high score suggests severe nerve injury (Spilker et al., 1997; Roth et al., 1998).

Data management

Clinical researchers filled out the complete clinical trial observation form accurately and in a timely manner. Data were recorded electronically by data managers using a double-data entry strategy. The electronic database was locked by the project manager after checking. All data were analyzed statistically by professional statisticians. Anonymized trial data will be published at www.figshare.com.

Statistical analysis

Data were presented as the mean ± standard deviation for normally distributed variables, or median values (P25, P75) for non-normally distributed variables. Student's *t*-tests or nonparametric Mann-Whitney *U*-tests were performed to compare the differences between normally distributed variables or non-normally distributed variables. For the analysis of biochemical test results, data were transferred to normal distribution and Hotelling's T2 tests were performed. Before entering variables into the regression model, centering predictor variables were performed to avoid nonessential collinearity. Binary logistic regression analysis was conducted to identify the association between variables or variable interactions and insomnia diagnosis after infarction. Multiple linear regression analysis was carried out to determine the correlation between variables or variable interactions and sleep-related scores, such as Epworth Sleepiness Scale scores, Pittsburgh Sleep Quality Index scores, Insomnia Severity Index scores, Morningness-Eveningness Questionnaire (Chinese version) scores and Fatigue Severity Scale scores by using the backward method. P values < 0.05 were considered statistically significant. SPSS 22.0 software (IBM, Armork, NY, USA) was used for statistical analyses.

Confidentiality

Clinical trial observation forms and informed consents were password-protected in the China Rehabilitation Research Center. The patient's identity will not be disclosed unless the law requires it. The findings will be published for scientific purposes without disclosing the patient's identity.

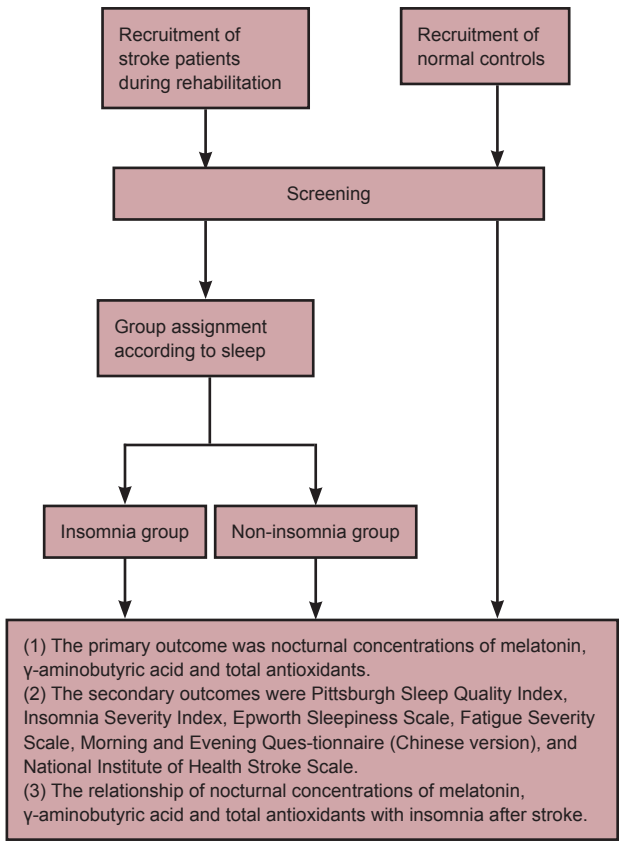

Figure 1 Clinical trial flow chart.

Table 1 Clinical information in the non-insomnia and insomnia groups

	Non-insomnia group	Insomnia group
n	25	25
Male [*n* (%)]	14 (56)	13 (52)
Age (year)	58.9±6.0	59.7±6.0
Months post infraction	10.80±3.96	10.32±4.17

Table 2 Comparison of sleep-related scales and National Institute of Health Stroke Scale scores between the non-insomnia and insomnia groups

	Non-insomnia group	Insomnia group
Epworth Sleepiness Scale score	3.36±2.02**	7.16±2.17
Pittsburgh Sleep Quality Index	4.00 (3.00–4.00)**	11.00 (9.50–14.00)
Morningness-Eveningness Questionnaire (Chinese version) score	58.36±5.96*	53.56±6.70
Insomnia Severity Index	2.28±2.21**	14.52±3.80
Fatigue Severity Scale score	11.84±1.65**	20.04±7.12
National Institutes of Health Stroke Scale score	7.08±2.45**	9.36±2.36

Data are presented as the mean ± SD for normally distributed variables, or median values (P25, P75) for non-normally distributed variables. Student's *t*-tests or nonparametric Mann-Whitney *U* tests were performed to analyze normally distributed variables or non-normally distributed variables. *$P < 0.05$, **$P < 0.01$, *vs.* the insomnia group.

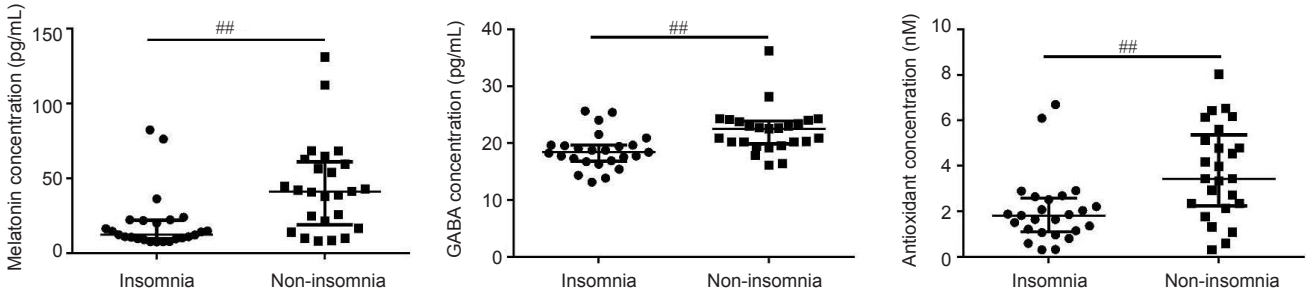

Figure 2 Nocturnal concentrations of melatonin, γ-aminobutyric acid (GABA) and total antioxidants in peripheral blood.
Melatonin and GABA concentrations were measured by ultra-performance liquid chromatography-mass spectrometry and the antioxidant concentration was measured by colorimetric antioxidant assay. Bars indicate the median and interquartile values. Hotelling's T2 test was performed for statistical analysis. ##$P < 0.01$.

Ethical requirements

The study protocol was approved by the Ethics Committee of China Rehabilitation Research Center on 20 March 2012. All protocols were performed in accordance with the Ethical Principles for Medical Research Involving Human Subjects in the *Declaration of Helsinki* (2013), formulated by the World Medical Association. The writing and editing of the article were performed in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) (**Additional file 1**). This trial was registered at ClinicalTrials.gov (identifier: NCT03202121). Written informed consents were provided by a legal representative of each patient after they indicated that they fully understood the treatment plan.

Results

Patient recruitment and data collection of the insomnia and non-insomnia groups is finished.

Clinical information of patients in the insomnia group and non-insomnia group

The characteristics of all participants in the study are listed in **Table 1**. There was no significantly statistic difference in gender, age, or months after infarction between the two

Table 4 Linear regression analysis of the relationship of GABA, antioxidants, melatonin, NHISS and factor interaction with sleep-related scale

Variable	*B*	SE	*β*	*t*	*P*
Epworth Sleepiness Scale score					
GABA	–0.210	0.090	–0.297	–2.341	0.024
Melatonin	–0.039	0.013	–0.389	–3.068	0.004
Pittsburgh Sleep Quality Index					
GABA	–0.414	0.108	–0.363	–3.821	< 0.001
Melatonin	–0.115	0.023	–0.960	–6.821	< 0.001
Melatonin × NIHSS	–0.026	0.006	–0.591	–4.196	< 0.001
GABA × NIHSS	0.069	0.045	0.146	1.534	0.132
Morningness-Eveningness Questionnaire (Chinese version) score					
GABA	0.405	0.216	0.240	1.879	0.067
Melatonin	0.150	0.045	0.627	3.309	0.002
Melatonin × NIHSS	0.025	0.012	0.381	2.012	0.050
Insomnia Severity Index					
GABA	–0.488	0.157	–0.286	–3.112	0.003
Melatonin	–0.254	0.033	–1.051	–7.725	< 0.001
Melatonin × NIHSS	–0.042	0.009	–0.634	–4.662	< 0.001
Fatigue Severity Scale score					
NIHSS	0.946	0.384	0.380	2.463	0.018
Melatonin	–0.110	0.048	–0.469	–2.280	0.027
Melatonin × NIHSS	–0.030	0.011	–0.478	–2.661	0.011

Melatonin × NIHSS, GABA × NIHSS, antioxidants × NIHSS, single biochemical factors and NIHSS were analyzed by liner regression. NIHSS: National Institutes of Health Stroke Scale; GABA: γ-aminobutyric acid; SE: standard error.

Table 3 Binary logistic regression for the relationship of biochemical factors, NIHSS and factor interaction with insomnia

Variable	*B*	*OR* (95% *CI*)	*P*
GABA	–0.494	0.610(0.438–0.849)	0.003
Melatonin	–0.127	0.881(0.815–0.953)	0.001
Melatonin × NIHSS	–0.023	0.977(0.960–0.995)	0.013
GABA × NIHSS	0.044	1.045(0.945–1.155)	0.392
Antioxidants × NIHSS	1.057	2.877(0.449–18.427)	0.265

Interactions such as melatonin × NIHSS, GABA × NIHSS, and antioxidant × NIHSS, was entered into Block 1, and single biochemical factors and NIHSS were entered into Block 2. The Hosmer–Lemeshow test *P* value is 0.576. NIHSS: National Institute of Health Stroke Scale; GABA: γ-aminobutyric acid; OR: odds ratio; CI: confidence interval.

groups ($P > 0.05$).

Differences in nocturnal concentrations of melatonin, GABA and total antioxidants in the peripheral blood of patients in the insomnia and non-insomnia groups

Results of UPLC-MS and colorimetric antioxidant assay showed that the nocturnal blood concentrations of melatonin, GABA, and total antioxidants in the insomnia group were lower than that in the non-insomnia group ($P < 0.01$; **Figure 2**).

Differences in sleep status of patients in the insomnia and non-insomnia groups

As shown in **Table 2**, patients in the insomnia group had significantly worse status assessed by related sleep scales. Of note, the Epworth Sleepiness Scale, Pittsburgh Sleep Quality Index, Insomnia Severity Index and Fatigue Severity Scale scores in the insomnia group were higher than in the non-insomnia group ($P < 0.01$). Additionally, patients in the insomnia and non-insomnia groups displayed significant differences in Morningness-Eveningness Questionnaire (Chinese version) scores, implying that the patients in the insomnia group can be represented as moderate evening types, whereas patients in the non-insomnia group can be represented as moderate morning types.

Relationship of nocturnal concentrations of melatonin, GABA and total antioxidants in peripheral blood of patients with insomnia after stroke

Results of UPLC-MS and colorimetric antioxidant assay showed that the nocturnal blood concentrations of melatonin, GABA, and total antioxidants in the insomnia group were lower than in the non-insomnia group ($P < 0.01$). Strikingly, the current data also showed an elevated NIHSS score in the insomnia group compared with the non-insomnia group ($P < 0.01$), indicating more severe infarction impairment in the insomnia group (**Table 2**).

The binary logistic regression analysis showed that GABA and melatonin levels in the blood might be associated with insomnia of patients with negative factors ($P = 0.003$ or 0.001), whereas the total antioxidant levels might not be an effective interaction factor (data not shown). Of note, the interaction between NIHSS and melatonin was associated with insomnia ($P = 0.013$), whereas interactions between NIHSS × GABA or NIHSS × antioxidant were not associated with insomnia (**Table 3**).

Based on the sleep-related scale scores, multiple linear regressions were achieved (**Table 4**). Interaction factors, such

as melatonin, GABA, and total antioxidants with NHISS, influenced the insomnia patients from different aspects. Both melatonin and GABA had negative correlations with ESS ($P = 0.024$, $P = 0.004$), PSQI ($P < 0.001$, $P < 0.001$) and ISI ($P = 0.003$, $P < 0.001$). Melatonin also had a positive correlation with the Morningness-Eveningness Questionnaire (Chinese version) score and a negative correlation with the Fatigue Severity Scale score ($P = 0.002$ and $P = 0.018$). In addition, regression analysis indicated that the melatonin × NIHSS interaction was significantly associated with the Pittsburgh Sleep Quality Index score ($P < 0.001$), Morningness-Eveningness Questionnaire (Chinese version) score ($P = 0.05$), Insomnia Severity Index score ($P < 0.001$) and Fatigue Severity Scale score ($P = 0.011$), but not with the Epworth Sleepiness Scale score ($P > 0.05$).

Discussion

Melatonin, an indole derived from serotonin, is a rhythmically secreted neurohormone produced mainly by the pineal gland (Claustrat et al., 2005; Atanassova et al., 2009; Lu et al., 2015). This feature makes melatonin an important sleep regulator that regulates circadian sleep and waking. Here, we collected blood samples from patients after middle cerebral artery infarction and directly measured the concentrations of melatonin via highly sensitive UPLC-MS. There was a significant decrease in nocturnal melatonin levels in the blood samples from insomnia patients. Regression analysis indicated a negative relationship between nocturnal melatonin levels and insomnia for post middle cerebral artery infarction patients. The analysis also suggested an influence of nocturnal melatonin levels on other aspects of insomnia, including the quality and patterns of sleep, circadian rhythm, severity of insomnia and fatigue levels.

All these results indicate that melatonin disturbance is an important factor for middle cerebral artery infarction patients who suffer from insomnia. Middle cerebral artery infarction is the most common subtype of stroke and is characterized by a poor prognosis (Ng et al., 2007). Furthermore, middle cerebral artery infarction has a clear blood supply area and its effects on sleep and other biochemicals are uniform without significant interference from other factors. Although the blood supply area of the posterior cerebral artery has a close relationship with the pineal gland, its main melatonin secreting region and blood supply area is regarded to be complicated because of the inclusion of the ascending reticular active system. In addition, the posterior cerebral artery infarction impacts the consciousness of patients, and may affect a patient's ability on finishing sleep-related scale assessments. As a result, the middle cerebral artery was explored as a starting point in the current study, which may allow us to exclude interference by other factors. Further studies of the posterior cerebral artery patients are required.

According to early reports, acute ischemic stroke patients, including anterior circulation stroke, extensive cortical stroke, and deep and lacunar strokes, show a significant decrease in nocturnal urinary melatonin excretion at day 3 post ischemic stroke, caused by melatonin rhythm disturbance or peak delay at the acute stage of stroke (Fiorina et al., 1996; Beloosesky et al., 2002; Atanassova et al., 2009). It was also reported that melatonin secretion pattern disturbances can be reverted to a normal pattern within 10 days post stroke (Beloosesky et al., 2002). However, there is still lack of reports on the dysregulation of melatonin or other neurotransmitters in infarction patients, especially those in the chronic rehabilitation stage.

Following the downregulation of melatonin levels in the blood of patients, sustained melatonin levels may partially contribute to the onset of insomnia in the rehabilitation phase of stroke patients. In addition, because melatonin is partially produced from serotonin in the brain, decreased melatonin levels in the blood of patient shown in the current study suggest decreased serotonin levels in the patients, which will be followed up in future studies. Our data also showed that middle cerebral artery infarction patients with insomnia complained of significantly higher NIHSS values compared with patients without insomnia. The NIHSS value is a reliable systematic tool to assess neurologic deficits in stroke-related patients (Spilker et al., 1997; Roth et al., 1998; Kasner, 2006), so an increased NIHSS value in insomnia patients may suggest a worse neurologic deficit induced by the middle cerebral artery infarction.

However, regression analysis in our study indicated that NIHSS itself and its synergic interaction with disturbed melatonin levels in the blood were markedly correlated with insomnia in these infarction patients. The interaction factor of NIHSS × melatonin level was negatively correlated with the quality of sleep (Pittsburgh Sleep Quality Index scores), severity of insomnia (Insomnia Severity Index scores), and fatigue level of patients (Fatigue Severity Scale scores), but positively correlated with the Morningness and Eveningness Questionnaire (Chinese version) score, indicating a change in sleep type after cerebral infarction may be a cause of insomnia after stroke. This change may be associated with the secretion of melatonin after cerebral infarction. To the best of our knowledge, this is the first demonstration of a clear association between the NIHSS × melatonin interactions and insomnia occurrence for post infarction rehabilitation of patients.

NIHSS is highly correlated with the size of ischemic area and severity of ischemic impairment in stroke patients (Spilker et al., 1997; Meuli, 2004; Kasner, 2006). These neurologic deficits may involve serious impairment of endogenous melatonin secretion from specific neurons, resulting in a decreased recovery of melatonin levels in rehabilitation patients and deficient quality of sleep or insomnia. However, early studies reported that melatonin in the blood was protective in a middle cerebral artery stroke rodent model (Sinha et al., 2001; Pei et al., 2003; Pei and Cheung, 2004). Thus, sustaining low blood levels of melatonin for middle cerebral artery infarction patients probably has low protection against ischemia injury. However, this study only reported limited observations, and the mechanism of such an underlying interaction are unknown. Currently, we are working on the etiology of insomnia for infarction patients to reveal this

interaction mechanism.

GABA is an inhibitory neurotransmitter and an important regulator that activates unused synapses and promotes the recovery and rehabilitation of patients after ischemic stroke (Morgan et al., 2012; Paik and Yang, 2014; Blicher et al., 2015). In this study, a functional decrease in GABA levels in the blood of patients after stroke reflected a disinhibition of synaptic activity and promotion of brain recovery. GABA levels in the blood of patients were decreased after subcortical lesion stroke, suggesting a decreased regulation of GABA to boost the recovery of brain function (Blicher et al., 2015). In this case, because patients in the insomnia group expressed a higher NIHSS, suggesting a worse impairment by cerebral infarction, the GABA level in the blood samples may result in a significant functional downregulation in the insomnia patients, which might assist stroke rehabilitation. A functional decrease of GABA in the blood may not only affect the sleep of patients but also be the cause of insomnia. However, it is only a partial explanation for the significance of GABA and insomnia post infarction.

We also studied the effect of antioxidants that have a critical role in the balance of oxidation by scavenging free radicals and limiting oxidative stress during neurological damage caused by ischemic stroke (Allen and Bayraktutan, 2009). Previous studies reported inconsistent results regarding changes in antioxidants in patients with acute ischemic stroke (Zimmermann et al., 2004; Aygul et al., 2006). According to our data, total antioxidant levels in the blood were decreased in patients of the insomnia group during the recovery stage post infarction. This suggests that patients in the acute ischemic stage and rehabilitation stage may have different regulatory mechanisms (Shekleton et al., 2010). In addition, melatonin contributes to antioxidant responses in ischemic stroke (Ritzenthaler et al., 2013); therefore, the decreased melatonin levels in the blood observed in this study may partially contribute to the decrease of total antioxidants in insomnia patients. Regression analysis indicated no association between the total antioxidant concentration and insomnia or sleep-related scores; therefore, a decrease in total antioxidants in the blood may be ascribed to the decrease of melatonin.

Although clinical evaluation dominates the final insomnia diagnosis, these valid and brief self-reports can expedite the evaluation of insomnia and promote lengthy routine clinical assessment (Morin et al., 2011). These self-report questionnaires provide a multidimensional measurement system to assess insomnia status. Based on the sleep-related scales, our data demonstrated that melatonin, GABA, and NIHSS as well as interactions between them affected insomnia symptoms. They also provide insight into the multidimensional aspects of insomnia, such as the quality and patterns of sleep, circadian rhythm, severity of insomnia and fatigue level of patients. Although our observations do not allow a conclusion regarding the cause-and-effect relationship, the data in this study demonstrate for the first time that melatonin, GABA, and total antioxidants are directly correlated with insomnia and other sleep disorders in patients with post middle cerebral artery infarction.

Recruitment of normal controls is ongoing, so it cannot completely explain the changes in nocturnal concentrations of melatonin, GABA and total antioxidants in the peripheral blood after stroke. Therefore, further data collection will provide an experimental basis for exploring the risk factors of sleep disorders after stroke.

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Conflicts of interest: *None declared.*

Research ethics: *The study protocol was approved by the Ethics Committee of China Rehabilitation Research Center.*

Declaration of patient consent: *The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.*

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Additional file:

Additional Table 1: *SPIRIT checklist.*

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