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Research Article

Therapeutic Effect Analysis of Tuina Manipulations in the Treatment of Insomnia and Itraq Quantitative Proteome Analysis

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ABSTRACT

Introduction: Insomnia, one of the most common mental disorders, not only affects the quality of life, but also damages physical and mental health. Therefore, it is very necessary to explore the molecular mechanism of insomnia and find some suitable treatments. At present, methods of using drugs to treat insomnia are not satisfactory due to lack of evidences and side effects. Hence, development of non-drug treatments is particularly important. Tuina manipulations, a Chinese massage method, has achieved certain therapeutic effects on injuries, rheumatism, neurological diseases and other types of diseases. We have treated patient with insomnia by Tuina manipulations, and obtained therapeutic effects indeed.

Methods: In the current study, assessments were performed using the Pittsburgh Sleep Quality Index (PSQI) and the insomnia severity index (ISI). iTRAQ (isobaric Tags for Relative and Absolute Quantitation) quantitative proteomics was used to analyze plasma samples taken from the Healthy Control (HC) group, insomnia patients group (before Tuina treatment, BTT) and insomnia-therapy group (after Tuina treatment, ATT) to identify the molecular correlation of insomnia.

Results: The results showed that the PSQI score and ISI score of the ATT group were significantly lower than those of BTT, and the difference was statistically significant. In addition, the proteomics results show that in BTT vs. HC, the expression of many immune-related and stress-related proteins is out of control, the expression of many immune-related and stress-related proteins were out of control, and it revealed that Tuina manipulations had the capacities to regulate expression of immune-related and stress-related proteins in ATT vs. BTT, suggesting that Tuina manipulations may improve insomnia by regulating immune-related and stress-related proteins. The proteomics verification results had been verified by commercial ELISA (Enzyme Linked Immunosorbent Assay.

Conclusion: All in all, our study not only found a good way to treat insomnia, but also provided a research foundation for improving insomnia.

Keywords: Insomnia; Tuina manipulations; Differentially expressed protein; Immunity; Stress

INTRODUCTION

Insomnia is one of the most common mental disorders in the world [1], usually manifested as difficulty in starting or maintaining sleep or early morning wake-up related to impaired daytime functioning [2]. According to the definition of the National Institutes of health, insomnia disorder includes sleep deficiency, sleep homeostasis, sleep fragmentation, insufficient sleep or impairment of sleep quality or quantity induced by a sleep disorder [3]. Sleep is a biological process that is important for optimal neurologic function, as well as systematic biology, including appetite regulation, metabolism, hormonal balance, immunity, and cardiovascular system [4,5]. The insufficient sleep will not only affect the life quality of the patients, but also do harm to psychological and physical health, which may further cause other conditions, such as anxiety, fatigue, depression, and cognitive decline, etc. [6-8]. Previous research data indicates that the prevalence of sleep disturbance ranges between 36% and 50% [9,10], and approximately 15% of people meet the criteria for insomnia in China [11]. Sleep disorders have been affecting a large number of people in the world and increasing in prevalence [12]. In addition, the prevalence of insomnia has imposed a huge economic burden on individuals and society [13-15]. Thus, it is

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really necessary to explore the molecular mechanism of insomnia and find suitable and effective treatments.

The treatments for insomnia disorder mainly consist of pharmacological and non-pharmacological therapies currently [16], among which hypnotics are the most commonly used pharmacological treatment methods as we know. However, long-term use of hypnotics is generally not recommended, due to lack of evidences and kinds of side effects [2]. These side effects mainly include tolerance, dependence, impaired cognitive function and poor quality of life [16,17]. Generally, non-drug therapies have a lower risk of drug-related side effects than most of drug therapies. Hence, patients are more inclined to choose non-drug therapies to improve their sleep conditions [18]. Moreover, the cognitive behavioral therapy for insomnia (CBT-I) which is a standard of non-pharmacological therapy for insomnia, has been underutilized for the scarcity of limited insurance coverage, CBT-I providers, non-responsiveness, and poor compliance [2,19,20]. Therefore, new complementary and alternative therapies with favorable benefit to risk ratio have been considered to be potential options for insomnia patients.

Tuina manipulations are standardized techniques including the use of hand or other parts of limbs to work on the lesion or to move limbs with the purpose of treatment or health care including soft tissue release manipulation and active joint manipulation [21]. It is used as an independent method and as an alternative to traditional and Western treatments [22,23]. Previous studies have indicated that Tuina manipulations has been successfully applied to various diseases such as neurological diseases, injuries, rheumatism and other types of diseases [24]. Tuina manipulations improve some common health problems by stimulating the acupressure points eliminate meridian obstruction and balance the flow of Qi and blood. Tuina manipulations have been used to assist in the treatment of muscle pain or stiffness for thousands of years [25-27], but there are not wide applications in insomnia. Studies have shown that the insomnia induced by joint deficit of the heart and spleen, treated with acupuncture, moxibustion and Chinese Tuina manipulations, provided significantly better results than that of patients treated with acupuncture and moxibustion alone [25]. It implies that Tuina manipulations may have a therapeutic effect on insomnia.

We tried to treat insomnia through Tuina treatment, and evaluated by the Pittsburgh Sleep Quality Index (PSQI) and the Insomnia Severity Index (ISI), and found that it does have a therapeutic effect. In order to explore the molecular mechanism of Tuina manipulations therapy for insomnia, we recruited a group of insomnia patients and healthy people, and collected plasma samples of Healthy Control Group (HC) and insomnia patients group (before Tuina treatment, BTT) and insomnia treatment group (after Tuina treatment, ATT).Subsequently, iTRAQ (isobaric Tags for Relative and Absolute Quantitation) quantitative proteomics and analyses were carried out. We found that Tuina manipulations may improve insomnia by regulating immune-related proteins and stress-related proteins. The proteomics verification results have been verified by ELISA (Enzyme Linked Immunosorbent Assay). All in all, our research not only found a good way to treat insomnia, but also provided a research foundation for improving insomnia. Here we report the results.

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MATERIALS AND METHODS

Ethical certification

This study was approved by the Hubei TCM Regional Ethics Review Committee (TCM), and the ethical approval identifier is HBZY2019-C23-01. All subjects' participation was voluntary and informed consent was obtained. We confirm that all experiments were performed in accordance with relevant guidelines and regulations.

Recruitment of participants

The subjects of the study were chronic insomnia patients recruited from Hubei University of Traditional Chinese Medicine, Hubei Provincial Hospital of Traditional Chinese Medicine through recruitment advertisements from July 2018 to December 2019. Finally, according to the diagnostic criteria of insomnia in previous study [18], 30 participants with a diagnosis of insomnia disorder and 30 control participants with good sleep quantity were recruited.

Inclusion criteria

(1) Meet the diagnostic criteria

(2) Age 18-70 years old

(3) PSQI score >5 points

(4) Have not taken sedative and hypnotic drugs in the past 3 months

(5) Volunteer to participate and sign the informed consent form

Exclusion criteria

(1) Those who do not meet the inclusion criteria

(2) Respiratory-related sleep disorders, restless legs syndrome, environmental sleep difficulties, short sleepers, drug-induced insomnia patients and patients with mental disorders

(3) Insomnia caused by systemic diseases such as pain or cough

(4) Patients with severe heart, liver, kidney, blood, and respiratory diseases

(5) Tumor patients

(6) Women during pregnancy

(7) Patients with cognitive dysfunction

(8) Patients who suffer from infectious diseases or skin infections that are damaged and are not suitable for manual treatment

Elimination criteria

(1) Those that do not meet the inclusion criteria and were mistakenly included.

(2) Those that take insomnia-related drugs by themselves or adopt other intervention methods during the study period.

(3) Those that have poor compliance during the research process and fail to follow the rules of the research protocol.

Falling off standard

(1) During the research process, poor compliance, due to various reasons, failed to complete the treatment according to the research protocol and withdrew halfway.

(2) Those that have serious adverse events or other special changes and are not suitable to continue to accept intervention.

Tuina treatment

Patients with insomnia were treated with head and face manipulation in Tai Chi Tuina (Figure S1) : (1) the patient took the supine position, the operator pushed and smeared the thumb abdomen along the governor's vein from Yintang (EX-HN3) to Shenting (DU24) for several times, then rubbed to Baihui (DU20) and pressed the Sishencong (EX-HN1); knead along the Yangbai (GB 14) spot until the Sishencong (EX-HN1), 3 ~ 5 times on the left and right respectively; push the thumb of both hands to the Taiyang (EX-HN5) from left to right along the upper orbital rim, rub the Taiyang (EX-HN5) with the thumb, extend the other four fingers into the occiput posterior, hold up the head, tap Anmian (EN-HN15), and use the four fingers and thumb alternately and symmetrically; (2) The operator pushed the thumb of both hands left and right along the upper orbital rim, and rubbed the Jingming (BL1), Yuyao (EX-HN4) and Sizhukong (SJ23) Kneading with two hands and four fingers: knead the side head along the Shaoyang (SanJiao), press the Sishencong (EX-HN1) with the thumb, and exert symmetrical force between the four fingers and the thumb. The middle and index fingers of both hands were in the shape of scissors, push and wipe up and down along the front and back of the tragus for several times, and click the corresponding acupoints on the ear with your thumb. Push the thumb of both hands to the Taiyang (EX-HN5) from left to right along the upper orbital rim, rub the Taiyang (EX-HN5) with the thumb, extend the other four fingers into the occiput posterior, hold up the head, tap Anmian (EN-HN15), and use the four fingers and thumb alternately and symmetrically; (3) Press the scalp on the midline of the head called the Governor Vessel; use four fingers to knead the cheeks on both sides, and press Daying (ST5), Jiache (ST6) car and Xiaguan (ST7) along the way. Push the thumb of both hands to the Taiyang (EX-HN5) from left to right along the upper orbital rim rub the Taiyang (EX-HN5) with the thumb, extend the other four fingers into the occiput posterior, hold up the head, tap Anmian (EN-HN15), and use the four fingers and thumb alternately and symmetrically; (4) After the palms of both hands were pushed several times from the forehead to the left and right along the cheek, the palms of both hands were closed, the fingers were naturally separated, the wrist joint was extended back, the head was rhythmically tapped several times with the side of the little finger, the four fingers of both hands were gently tapped and bounced on the head, the shoulder well was pinched for 1 ~ 2 times, and the patient's shoulder was tapped to end the manipulation.

The treatment took 5 days as a course of treatment. After 2 days of rest, the next course of treatment was carried out for 4consecutive courses of treatment.

Assessment of sleep disturbance

Pittsburgh Sleep Quality Index (PSQI) and Insomnia Severity Index (ISI) were used to evaluate sleep disorders.

PSQI is a self-assessment questionnaire on sleep quality in the past month, which consists of 19 self-assessment questions. These questions are divided into seven components, and the scores of each component range from 0 to 3. Then add the scores of the seven components to obtain a global PSQI score, which ranges from 0 to 21. The higher the score, the worse the sleep quality [28-45]. Buysse et al. reported that the english version of PSQI is highly reliable. ISI uses seven questions to measure sleep maintenance difficulties, satisfaction with the current sleep mode, interference with daily functions, obvious damage caused by sleep problems and attention caused by sleep problems [46,47]. The seven items are scored on a scale of 0 to 4, with a total score ranging from 0 to 28. The higher the score, the more serious insomnia [47].

Statistical analysis

Collected data were analyzed using SPSS version 23.0 (SPSS Inc., Chicago, IL). The data are presented as the means ± standard deviation, and $p<0.05$ was set as the threshold for statistical

significance. The data conforming to the normal distribution are subject to T-test, and the data not conforming to the normal distribution are subject to rank sum test.

Sample collection

There were three groups of experimental samples including plasma samples of Healthy Controls (HC), plasma samples of insomnia patients Before Tuina Treatment (BTT), and plasma samples of insomnia patients After Tuina Treatment (ATT). The fasting plasma samples were taken at 11:00, 1 h after participants relaxed in a temperature-controlled (25°C) room. Centrifuge immediately after collecting blood on ice in tubes with K3-EDTA or Corvac. The plasma samples were obtained after centrifugation, and stored at -80°C until further use.

Protein enrichment

The frozen plasma samples from the three groups (BTT (n=9), ATT (n=9) and HC (n=9)) were thawed. Subsequently, the plasma from three persons in the same group was randomly mixed together at a ratio of 1:1:1, and the three experimental groups were mixed in this way to avoid individual errors.

According to the instructions of manufacturer, the pooled plasma samples were pretreated by Proteo-Miner™ protein enrichment kit (Bio-Rad, Hercules, CA, USA) to remove high-abundance proteins and collect the final eluate. The collected protein solution after depletion was transferred to another centrifuge tube, to which a 4-fold volume of cold acetone was added and kept at -20°C for 10 h. The acetone protein precipitant was collected through centrifugation, then dried in air and re-dissolved in 8 M urea/100 mM Triethylamonium Bicarbonate (TEAB) (pH 8.0). The Dithiothreitol (DTT) was added at a final concentration of 10 mM for the reduction reaction for 30 min at 56°C, and then the iodoacetamide was added to a final concentration of 55 mM for an alkylation reaction for 30 min in the dark. A Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) was used to measure the concentration of protein samples. In order to evaluate the effect of pretreatment, quality control was performed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

iTRAQ quantitative proteomics analysis

We mainly referred to previous experimental methods and made some adjustments [48]. Approximately100 μg protein from each sample was digested by trypsin. We diluted the protein solution 5 times by100 mM tetradecyltrimethylammonium bromide (MTEAB), next added trypsin at a ratio of trypsin: protein of 100:1 for enzymatic hydrolysis at 37°C for 12-16 h. After trypsin digestion, the resulting peptide was desalted with C18 columns, dried in vacuum, and redissolved with 0.5 M TEAB. The digested samples were labelled with an iTRAQ reagents-8 plex kit (SCIEX)according to the instructions of manufacturer as follows: three mixed groups of HC were labelled with a mass of 116-1,116-2,116-3three mixed groups of BTT were labelled with a mass of 115-1,115-2,115-3 three mixed groups of ATT were labelled with a mass of 114-1,114-2,114-3. Combine the three iTRAQ-labeled peptide samples with the same serial number (116-1, 115-1, 114-1; 116-2, 115-2, 114-2; 116-3, 115-3, 114-3) to conduct three independent experiments.

Next, the polypeptide solution was added to Durashell C18 column (5 μm, 100 Å, 4.6 x 250 mm) by an Ultimate 3000 HPLC system (Thermo DINOEX, USA). The peptides segment was separated by increasing the Concentration of Acetonitrile (CAN) at the flow rate of 1 ml/min under alkaline conditions. The fractions were collected every 1 min, and a total of 42 secondary fractions were collected. Finally, the collected fractions were combined into 15 fractions and dried by vacuum centrifugation.

LC-MS/MS analysis

The TripleTOF 5600 plus liquid chromatography-mass spectrometry (SCIEX, USA) in combination with an Eksigent nanoLC system (SCIEX, USA) was used to capture the mass spectrometry (MS) data. These polypeptide samples were dissolved in the solution comprising 2% acetonitrile/0.1% formic acid, and subsequently transferred to the C18 capture columns (5 μm, 100 μm x 20 mm). Gradient elution was implemented in a C18 analytical column (3 μm, 75 μm x 150 mm) with a 90 min gradient at a flow rate of 300 nl/min (mobile phase A comprising 0.1% (v/v) formic acid and 2% (v/v) acetonitrile; mobile phase B comprising 0.1% (v/v) formic acid and 80% (v/v) acetonitrile). The Information Dependent Acquisition (IDA) was used to scan the 1st-order mass spectra (MS1) with an ion accumulation time of 250 ms, while the 2nd-order mass spectra (MS2) of 30 precursor ions were collected with an ion accumulation time being 50 ms. The spectra of MS1 and MS2 were captured in the range 350-1,500 m/z and 50-2,000 m/z, respectively. In this iTRAQ project, we set the energy of the ion fragmentation at 35 ± 5 eV, while the parent ion dynamic exclusion set was set to half of the peak time (about 15 s).

Data analysis

Protein Pilot V4.5 software was employed for proteome identification and iTRAQ quantification. For proteome identification, Uniprot/Swiss-Prot database of human species (downloaded in June 2020) was used. Other parameters were set as follows: instrument, TripleTOF 5600; cysteine alkylation, iodoacetamide; digestion, trypsin; biological qualifications including ID focus and trypsin digestion; the Quantitate, Bias Correction and Background Correction was checked for protein quantification and normalization. Only the proteins with at least one unique peptide and unused value ≥ 1.3 (credibility ≥ 95%) was used for the further analyses.

The pairwise comparisons method between biological replicates was standardized as the ratios, and the smallest ratio was used as p-values to explore the Differentially Expressed Proteins (DEPs) under pair t-test. For the determination of DEPs, the Fold Changes (FC) were calculated as the average comparison pairs among biological replicates, and the proteins with FC>1.2 and $p<0.05$ were considered to be the DEPs.

Functional analysis

Identified DEPs were submitted to Gene Ontology (GO) [49] Terms (http://geneontology.org/) for classification, by which the DEPs could be assigned into three branches: Molecular Function (MF), Biological Process (BP), and Cellular Components (CC). In addition, the Pathway enrichment analysis was implemented by the Kyoto Encyclopedia of Genes and Genomes (KEGG) [50] (https://www.kegg.jp/). The enrichments were checked statistically with Fisher's exact test, and those with p-values ≤ 0.01 were considered to be statistically significant.

Enzyme Linked Immunosorbent Assay (ELISA)

Candidate protein (C5a, C4, C5b-9) levels were measured using Human Complement Fragment 5a (C5a) ELISA KitHuman

(Cusabio, Wuhan, China), Human complement 4 (C4) ELISA Kit (Cusabio, Wuhan, China), TCC C5b-9 (Terminal Complement Complex C5b-9) ELISA Kit (Elabscience, Wuhan, China) according to the instructions of manufacturer, respectively.

RESULTS

Participant characteristics

A total of 60 eligible subjects were recruited in this study, 4 of whom were dropped out, and the dropout rate was 6.7%. Among them, there were 29 people in the massage group and 27 people in the healthy control group, a total of 56 people, including 19 males and 37 females. Subsequently, we performed massage therapy on patients with insomnia, and the results showed that after the Treatment of Insomnia (ATT), the PSQI score and ISI score were significantly reduced, compared with the patients Before Treatment (BTT), the difference was statistically significant ($P \leq 0.01$), indicating that massage is effective for the treatment of insomnia (Figures 1A and1B). In order to explore the relevant mechanisms in depth, for patients with insomnia, we took plasma sample before and after the Tuina treatment (BTT, ATT). For the HC group, we took plasma sample without taking any treatment measures, and carried out subsequent experiments using samples from the three groups.

In the iTRAQ analysis and the ELISA analysis, the gender distribution and average age between the two groups were similar.

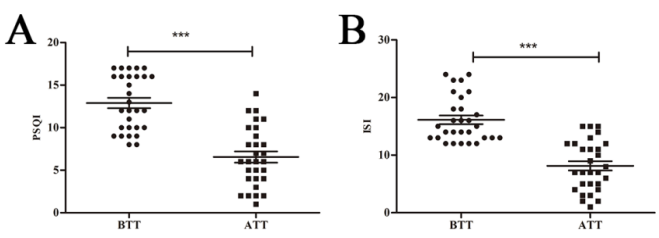

Figure 1: The clinical effect of Tuina manipulations in the treatment of insomnia. (A)Comparison of PSQI scores before and after Tuina treatment. (B)Comparison of ISI scores before and after massage treatment. (BTT, before Tuina treatment, insomnia patient group; ATT, after Tuina treatment, insomnia-therapy group ***P<0.001).).

The summery of iTRAQ-based proteomics analysis

In this part of the study, we randomly selected 9 BTT and 9 ATT plasma samples from 29 recruited insomnia patients, and 9 plasma samples from 27 recruited healthy controls. Subsequently, the plasma from three persons in the same group was randomly mixed together at a ratio of 1:1:1, and the mixed 9 standard samples (HC-1, HC-2, HC-3; BTT-1, BTT-2, BTT-3; ATT-1, ATT-2, ATT-3) were identified by mass spectrometry.

Before analyzing the proteins identified from the high throughput assay, we first looked at the quality of the data that obtained from the mass spectrometry. For the same we looked at these features such as the distribution of unique peptide number, peptide length, the distribution of coverage identified, and repeatability using parameters such as coefficient of variation. Unique peptides are defined as the peptides that are found only for one protein. From the presence of this type of peptide, the existence of the corresponding protein can be uniquely determined. It shows the coordinate distribution of the number of unique peptides contained in all the proteins identified in this assay (Figure 2A). The x-axis is the number of unique peptides that contained in the protein, the left y-axis depicts the number of proteins corresponding to the x-axis and the right y-axis corresponds to the ratio of total protein. For example, there are 325 proteins with 2 as the unique number of peptides, which is 80.65 % of the total number of proteins obtained. To increase the number of unique peptides, this inference could be further made. Next, the distribution of peptide length was analyzed (Figure 2B). As it could be seen from the figure, the average length of the polypeptide identified in the assay was 14.43, which was within a reasonable range of the peptide length. The figure also showed that the length of peptide was mainly concentrated between 7and 19 with a length of 11 peptides number showing maximum number of peptides. For an identified protein, the more peptides that support the protein, the higher the confidence of the protein. Therefore, the identification coverage of the protein indirectly reflected the overall accuracy of the identification results (Figure 2C). The different colored sectors in the pie chart represented the percentage of proteins with different ranges of determined coverage. It was clear from the figure that 73.20% of the identified proteins had equal to or more than 10% of the peptide coverage, and 58.06% had equal to or more than 20% of the peptide coverage.The520proteins were classified through Blast2go to assess Gene Ontology (GO) enrichment. Based on different biological process, these grouped proteins were as follows: metabolic process (8.18%), immune system process (4.24%), cellular component organization or biogenesis (5.72%), response to stimulus (7.66%), biological regulation (8.29%). The common proteins are mainly involved in metabolic, structural, and regulating processes (Figure 2D).

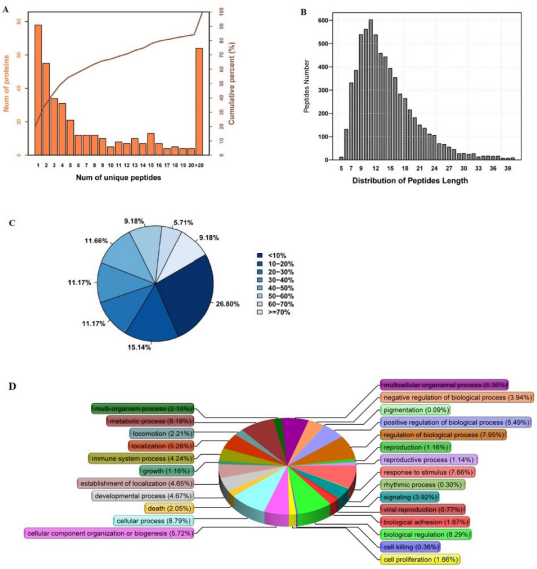

Figure 2: Summary of iTRAQ-Based proteomic analysis for plasma protein.(A) Distribution of the number of unique peptides obtained for all the proteins identified in this assay. (B) Peptide length distribution map of the identified peptides. (C) Sectors of different colors in the pie chart representing the percentage of protein with different ranges of identified coverage. (D) GO enrichment analysis of 520 plasma protein *via* Blast2go. Proteins were classified by biological process (BP) (HC, the healthy control group; BTT, before Tuina treatment; ATT, after Tuina treatment).

Exploration of Differentially Expression Proteins (DEPs)

We consider the cut-off value of all iTRAQ ratios as a 1.2-fold

change, that is, ratios>1.2 or <0.80, and classify proteins as up-regulated or down-regulated, respectively.

In BTT vs. HC, 97 DEPs were screened successfully, of which 55 were up-regulated and 42 were down-regulated (Figure 3) (Table S1). It suggested that in ATT vs. HC, there were 46 up-regulated and 46 down-regulated DEPs (Table S2). In ATT vs. BTT, 63 DEPs were identified, including 23 up-regulated and 40 down-regulated DEPs (Table S3). In addition, there were 17 common DEPs were identified between all three comparisons (Figure 3).

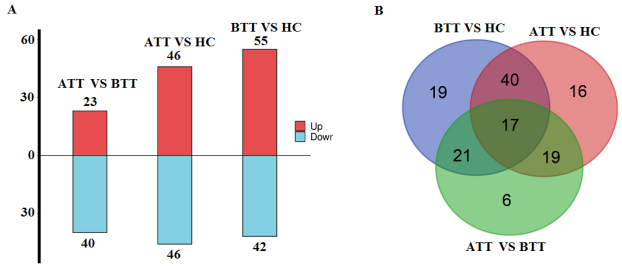

Figure 3: Exploration of the differentially expressed proteins (DEPs). (A) Histogram of Differential Proteins. Proteins with a fold change >1.2 and the Benjamini-Hochberg adjusted p value <0.05 consistent in DeSeq and t-tests are called differential proteins. (B) The number of exclusive and common DEPs between different groups. Venn diagram is created using online Venn diagram maker (HC, the healthy control group; BTT, before Tuina treatment; ATT, after Tuina treatment).

Functional analysis of DEPs

To identify the primary functions in which the DEPs (Figures 4A-4F) were involved, GO (Gene Ontology) enrichment analysis was carried out. There were 55, 63 and 47 terms were enriched successfully in the BTT vs. HC, ATT vs. HC and ATT vs. BTT comparisons (P<0.01) (Tables S4-S6; Figures 4B, 4D and 4F), respectively.

In BTT vs. HC, it was found that multiple enriched terms were associated with immunity, including "regulation of humoral immune response" (GO:0002920), "regulation of complement, activation" (GO:0030449), "inflammatory response (GO:0006954)", "regulation of acute inflammatory response (GO:0002673)", "acute inflammatory response" (GO:0002526), "regulation of immune effector process" (GO:0002697) and "regulation of inflammatory response (GO:0050727)", "complement activation, alternative pathway" (GO:0006957), "immune effector process" (GO:0002252), "regulation of immune system process" (GO:0002682), "regulation of complement activation, lectin pathway" (GO:0001868), "negative regulation of complement activation, lectin pathway" (GO:0001869), "activation of plasma proteins involved in acute inflammatory response" (GO:0002541) and "complement activation"(GO:0006956) (Table S4, Figure 4B). It suggested that insomnia might be associated with disorder of the immune system. Moreover, six terms were involved in stress were found including "response to wounding" (GO:0009611),"response to stress" (GO:0006950), "regulation of response to external stimulus" (GO:0032101), "response to external stimulus" (GO:0009605), "response to stimulus" (GO:0050896) and "regulation of response to stress" (GO:0080134), suggesting that insomnia might be involved with response of stress.

In ATT vs. HC, it was found that seven enriched terms were associated with immunity, including "inflammatory response" (GO:0006954), "acute inflammatory response" (GO:0002526), "regulation of acute inflammatory response" (GO:0002673), "regulation of immune system process" (GO:0002682), "regulation of complement activation, lectin pathway" (GO:0001868), "negative regulation of complement activation, lectin pathway" (GO:0001869), and "regulation of humoral immune response" (GO:0002920),and three terms were involved in stress were found, including "regulation of response to external stimulus" (GO:0032101), "response to stress" (GO:0006950), "response to external stimulus" (GO:0009605) (Table S5, Figure 4D).

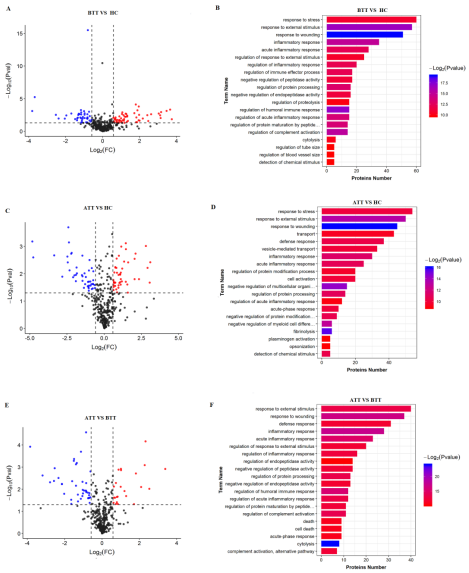

Figure 4: Functional analysis of differentially expressed proteins (DEPs). (A), (C) and (E) Volcano plots of DEPs from BTT vs. HC, ATT vs. HC and ATT vs. BTT. Each dot represents a specific protein, and the black dots represent different proteins that are not important. The blue and red dots indicated significantly up-regulated and down-regulated DEPs respectively. (B), (D) and (F) GO analysis of DEPs from BTT vs. HC, ATT vs. HC and ATT vs. BTT, respectively. The top 20 terms are presented. GO, Gene Ontology; DEPs, differentially expressed proteins. (HC, the healthy control group; BTT, before Tuina treatment; ATT, after Tuina treatment).

In ATT vs. BTT, it was found that multiple enriched terms were associated with immunity, including "inflammatory response" (GO:0006954), "regulation of humoral immune response" (GO:0002920), "acute inflammatory response" (GO:0002526), "regulation of complement activation" (GO:0030449), "regulation of acute inflammatory response" (GO:0002673), "regulation of inflammatory response" (GO:0050727), "complement activation, alternative pathway" (GO:0006957), "regulation of immune system process"(GO:0002682), "regulation of immune effector process" (GO:0002697), "adaptive immune response" (GO:0002250), "leukocyte mediated immunity" (GO:0002443), "activation of plasma proteins involved in acute inflammatory response" (GO:0002541), "complement activation" (GO:0006956), "humoral immune response mediated by circulating immunoglobulin" (GO:0002455) and "complement activation, classical pathway" (GO:0006958) (Table S6, Figure 4F). It strongly suggested that the Tuina treatment might have the capacities to affect the immune system and improve the insomnia by cure the disorder of immune system. Moreover, four terms involved in stress were

found including "response to external stimulus" (GO:0009605), "regulation of response to external stimulus" (GO:0032101), "regulation of response to stimulus" (GO:0048583), "positive regulation of response to stimulus" (GO:0048584), suggesting that the Tuina treatment could also improve the sleep quality by affecting the response to stress.

Validation of protein identification and quantification by ELISA

We performed ELISA to analyze the expression levels of C4, C5 and C5b-9 to confirm the results of iTRAQ-labeled LC-MS/MS analysis. Compared with the HC group, the expression levels of C4, C5 and C5b-9 in the BTT group and ATT group were decreased. Compared with the BTT group, the expression levels of C4, C5 and C5b-9 were increased in the ATT group (Figure 5).The result of ELISA supported the result of iTRAQ, which proved that our experiment was very reliable.

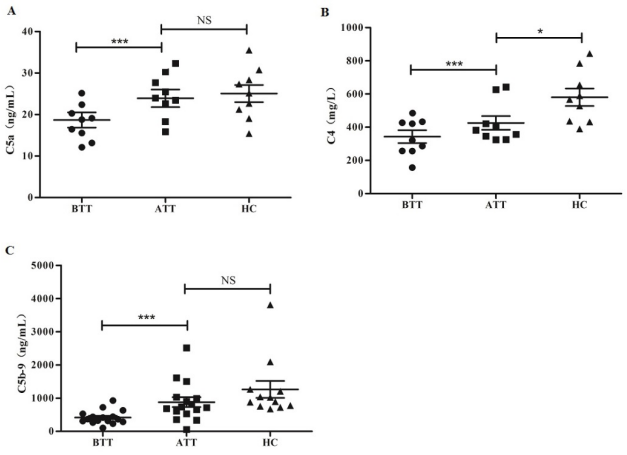

Figure 5: Detect the expression levels of C5a, C4, C5b-9 in HC, BTT andATT groups by ELISA.HC, the healthy control group; BTT, before Tuina treatment, insomnia patient group; ATT, after Tuina treatment, insomnia-therapy group *P<0.1, **P<0.05, ***P<0.001; NS, no significances.

DISCUSSION

Sleep is an important part of our daily life and one of the important predictors of people's health [28]. It is a complex biological process which is necessary for optimal neurologic function, as well as systematic biology including metabolism, appetite regulation, immunity, hormonal balance and cardiovascular system [4,5]. Good sleep quality has the capacities to enhance the immune defense of the bodies, while poor sleep status does harm to physical and mental health [29]. Sleep disorders have affected many people in China and even in the world [12]. For example, surveys conducted in Tianjin in 2019 and Liaoning Province in 2013 showed that the incidence of sleep disorders was 13.2% and 11.59%, respectively [30]. Insomnia is a huge problem of the public health, so people have been seeking complementary and alternative therapies all the time, due to the side effects of drug therapy [2,18]. Due to its wide availability, practicality and high compliance, Tuina manipulations therapy has been an important treatment in China for thousands of years to help treat muscle pain or stiffness in the body [25, 27].

In this study, we used Tuina manipulations therapy to treat patients with insomnia and achieved good results. After insomnia patients were treated with Tuina therapy, the PSQI score and ISI score were significantly reduced compared with them before the treatment, and the difference was statistically significant. In order to explore the molecular mechanism of Tuina manipulations therapy for insomnia, we used iTRAQ quantitative proteomics technology for the first time and found that the immune-related proteins and stress-related proteins of patients with insomnia have undergone significant changes through functional analysis of DEPs.

The sleep and circadian systems have strong regulatory effects on immune functions [31].Innate immunity is the first line of defense against tissue damage and microbial infection in the animals [32]. The discovery of reciprocal connections between the central nervous system, sleep and the immune system has suggested that sleep could promote immune defences and the afferent signals from the immune cells might improve sleep. One proposed mechanism by which sleep provides a vital advantage for survival is to support the neurointegrated immune system, which may anticipate the threat of injury and infection [33]. The interaction between sleep and the biological mechanisms of inflammation highlights the impact of sleep dysfunction on the risk of inflammatory diseases. Deciphering the mechanisms is good for development of treatments which regulate inflammation, and improve sleep health [34]. Moreover, the chronic sleep disturbances might be the cause or the consequence of other known triggers of low-grade inflammation, including obesity [35], circadian disruption [36], detrimental lifestyle habits [37], physical inactivity [38], psychosocial influences [39], stress [40], and low socioeconomic status [41]. These are all factors that should be controlled for in studies linking short or disturbed sleep and inflammatory processes. In BTT vs. HC, ATT vs. HC and ATT vs. BTT, multiple DEPs were associated with immunity. On the one hand, it supports the relationship between insomnia and immune disorders. On the other hand, it suggests that Tuina manipulations may affect the immunity and thus the therapeutic effect.

Naturalistic stress exposure, a precipitant of insomnia [42], has long been suggested that insomnia might begin with an organic predisposition towards poor sleep and wake-promoting hyperarousal [43]. Truely, insomnia disorder is always induced by the stressed events, the hyperarousal could interfere with sleep and lead to chronic insomnia in some persons [44]. The literature on stress and sleep is very vast and rich, and its comprehensive analysis deserves further review. In BTT vs. HC, ATT vs. HC and ATT vs. BTT, multiple DEPs were associated with stress. On the one hand, these findings support that insomnia is related to response to stress. On the other hand, it suggests that Tuina manipulations might influence response to stress, thereby treatment effect.

CONCLUSION

All in all, we show here that the expressions of the immune-related proteins and stress-related proteins of patients with insomnia are different between the insomnia group and the insomnia treatment group. It is speculated that Tuina manipulations may regulate the immune-related proteins and stress-related proteins of patients with insomnia, thereby improving insomnia. These results indicate that Tuina manipulations may have the effect of treating insomnia, which will provide a new idea for the future drug-free treatment of insomnia.

AUTHOR CONTRIBUTION

Yan Zhao conceived of the study, and participated in its design. Jing Zhou participated in the design of the study and coordination and drafted the manuscript. Biwei Cao participated in the design of the study,assessed treatment effectiveness and collected clinical data. Meng Wei participated in the design of the study and performed the statistical analysis. Yuan Xiong interviewed patients before and after treatment and collected clinical data. Wan Liu recruited and screened eligible participants from outpatient department, and assigned patients to either massage group or control group. Li Zhu participated in trial design and helped to prepare the manuscript. All authors read and approved the final manuscript.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interest.

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