

Original Article

Effects of Mongolian medical warm acupuncture on model rats with insomnia based on positron emission tomography-computed tomography (PET-CT)

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Abstract: Objective: Insomnia is one of the most common sleep disorders, with a very high morbidity. Mongolian medicine is characterized by a unique theory, presenting favorable therapeutic effects in the treatment of insomnia. In Mongolian medicine, warm acupuncture is ubiquitously applied for treatment of insomnia. It can invigorate bodily functions, regulate qi, blood, and somatostatin, strengthen immunity, and prevent and treat multiple diseases. The current study aimed to explore the effects of Mongolian medical warm acupuncture on cerebral glucose metabolism in insomnia rats using the PET-CT technique. Materials and methods: Rats were injected with para-chlorophenylalanine (PCPA) to establish the insomnia model. Rats were randomly divided into the normal group, model group, and warm acupuncture group, which received tail intravenous injections of ¹⁸F-FDG for scanning imaging of PET/CT. Expression of NDRG2 was detected with Western blotting. Differential expression of microRNAs in the brain tissues of the insomnia rats, before and after Mongolian medical warm acupuncture, was detected for qPCR verification. Plasmids were transfected to neurocytes of the rats for dual-luciferase reporter assay examination. Expression of NDRG2 was detected with the immunohistochemical method. Relevant cytokines and neurotransmitters were detected with ELISA. Results: Glucose metabolism in the Mongolian medical warm acupuncture group was strengthened, compared with that in the model group. Present results showed significant differences in the effects of Mongolian medical warm acupuncture, which plays a promoting role in cerebral function activity of model rats with insomnia via activating most regions inhibited in the brain. Chip data analysis found that expression of 156 miRNAs in the rats treated with Mongolian medical warm acupuncture was obviously altered. Of which, levels of miR-181a were increased by 3.2 times, compared with those in the model group. In silico analysis with TargetScan, PicTar, and miRanda showed that miR-181a and NDRG2 might have a target regulation relationship. Results of luciferase assay in 293T cells indicated no significant changes in the MUT-NDRG2-3'UTR group, while fluorescence intensity in the WT group was decreased significantly, following addition of miR-181a mimics. Western blotting results indicated that expression of NDRG2 in neuronal cells of the model rats with insomnia was significantly downregulated at 72 hours, after addition of miR-181a mimics, compared to that in the scramble group ($P < 0.01$). Administration of warm acupuncture reduced volume shrinkage. Rod-like fusion occurred in NDRG2-positive cells of the rats, while expression of NDRG2, particularly in the neuron cytoplasm, was downregulated, compared to that in model group. Levels of IL-1, IL-2, IL-6, and TNF- α in the warm acupuncture group increased significantly, compared with those in the model group ($P < 0.05$). Warm acupuncture significantly increased levels of GABA and reduced levels of Glu, compared with the insomnia model group. Conclusion: Mongolian medical warm acupuncture plays a promoting role in glucose metabolism in the brain of the model rats with insomnia. It upregulates levels of miR-18 and reduces NDRG2 levels, providing an academic basis for modern development of the traditional ethnic medicine.

Keywords: Mongolian medical warm acupuncture, PET-CT, insomnia, immediate central mechanism

Introduction

Insomnia indicates an unfavorable quality or amount of sleep, failing to meet normal physiological needs. It influences social function, as

well. It is currently one of the most common sleep disorders, with a very high morbidity [1]. According to the World Health Organization, about 1/3 of the population suffers from sleep disorders. The percentage of people with vari-

ous types of sleep disorders in China is significantly higher than 27%, the average percentage in the world [2].

In clinical treatment, modern medicine mainly uses hypnotics, while medication produces many side effects which influence long-term therapeutic effects. Likewise, long-term administration may lead to addiction and tolerance [3]. In Mongolian medicine, it is believed that imbalances of Sangen and the prevalence of Haoyi are basic causes of the disease. Negative emotions, such as tension, worry, horror, depression, and anxiety, as well as social environment, diet, daily life, and motion, are external causes. The major treatment principle comprises alleviation of Haoyi and regulation of the balance of Sangen [4]. Warm acupuncture is one of the most common methods for treatment of insomnia in Mongolian medicine. It aims to warm and smooth channels, regulate qi, blood, and somatostatin, strengthen immunity, and prevent and treat diseases. It is generally accepted by patients due to such characteristics as high efficacy, safety, absence of side effects, simplicity, and absence of drug dependence [5, 6].

Since the 1990s, with the development and application of neuroimaging techniques, acupuncture cerebral function imaging has emerged as a modern research approach explaining the central mechanisms of acupuncture. In the modular theory of brain function research, various cognitive functions are associated with a certain region (module) in the brain. Many studies have attempted to scientifically explain the effects of acupuncture along the meridians by observing changes in cerebral region functions arising from acupoint acupuncture, with the assistance of functional magnetic resonance imaging (fMRI), positron emission positron emission tomography-computed tomography (PET-CT), and single photon emission computed tomography [8]. Using a para-chlorophenylalanine (PCPA)-induced rat model, this study explored the effects of Mongolian medical warm acupuncture on glucose metabolism within the brains of the insomnia rats, using the PET-CT technique. The aim of this study was to provide a visual basis for studying the central mechanisms of selecting acupoints along channels.

Materials and methods

Mongolian medicine Model MY-I electric-heating needle warmer (Patent No ZL 2011 2 0058078.0) (**Figure 1A, 1B**) and sterile Mongolian medicine silver needles were produced by Inner Mongolia Medical University (Huhhot, Inner Mongolia, China). SIEMENS Inveon MM small animals PET/CT were purchased from Siemens AG Ltd. (Munich, Germany). Isoflurane, batch no. 20160204, was obtained from RWD Life Science Co., Ltd. (Shenzhen, Guangdong, China). A total of 60 clean SD male rats, weighing 160-180 g, were provided by the Laboratory Animal Center of Inner Mongolia University (Huhhot, Inner Mongolia, China). Rats were intraperitoneally injected with para-chlorophenylalanine (PCPA) (Lanbote, Beijing, China) at a dose of 300 mg/kg at 8:30-9:00 am for 2 consecutive days. Rat models of insomnia were adaptively raised for 7 days. PCPA was dissolved with normal saline (pH: 7-8) for suspension as per 1 mL/100 g. Evaluation of PCPA rat models of insomnia included obvious and frequent activity, abnormal sensitivity to such stimulants as sound and light, increased excitability, strengthened aggressiveness, gray excrement, circadian rhythm disorders, and continuous daytime motion occurring in the rats 28-30 hours after receiving the second intraperitoneal injection of PCPA. These factors suggest that modeling was successfully established [9]. SD rats were randomly divided into three groups, the normal group, model group, and warm acupuncture group, with 15 rats in each group. Normal group: Rats were intraperitoneally injected with normal saline at a dose of 0.1 mL/kg, for two consecutive days in the morning on each day, after being adaptively raised for 7 days. They were fed twice, for 1 hour each day, and received no other stimulation. Model Group: Rats were intraperitoneally injected with PCPA (300 mg/kg) in the morning for 2 consecutive days. This was for the establishment of rat models of insomnia after being adaptively raised for 7 days. No other stimulation was administered. Warm acupuncture group: Rats were intraperitoneally injected with normal saline at 8:30-9:00 a.m. each day, at a dose of 0.1 mL/kg for 2 consecutive days, after being adaptively raised for 7 days. Rats were stimulated with warm needles at the Dinghui acupoint, Haoyi acupoint, and Xin acupoint, for 15

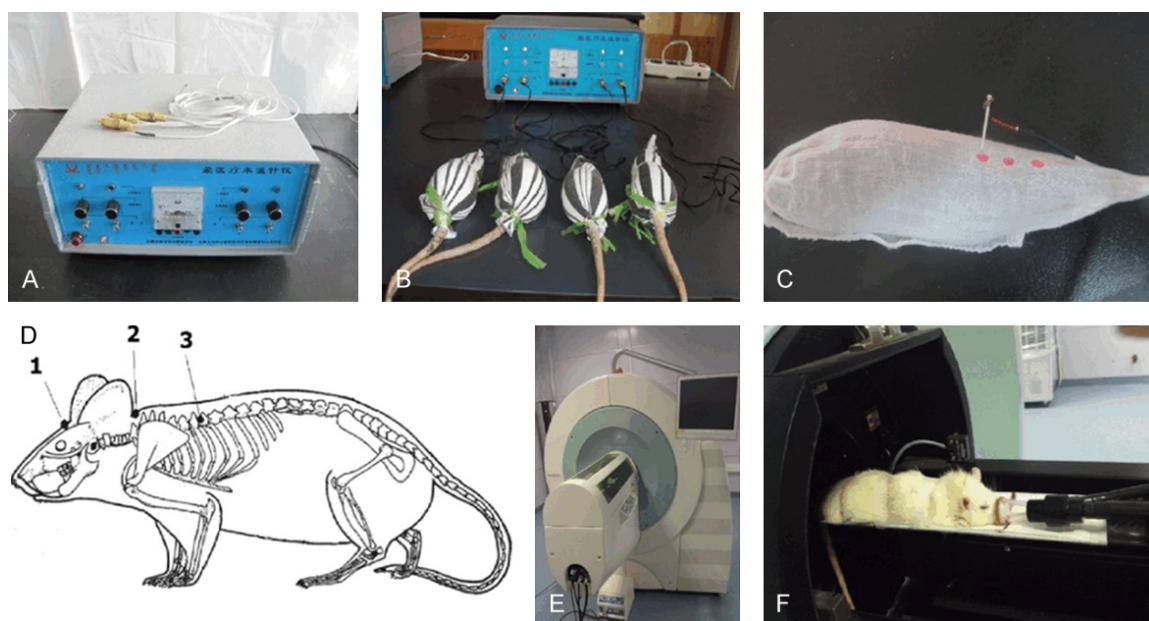


Figure 1. Experimental equipment for Mongolian medical warm acupuncture and PET-CT. A. Mongolian medicine Model MY-I electric needle warmer. B. Diagram for operating with electric needle warmers and sterile Mongolian medicine silver needles. C. Positions of Dinghui Acupoint, Haoyi Acupoint, and Xin Acupoint on the acupuncture cloth bag. D. Anatomical drawing of acupoint locations of rats. E. SIEMENS Inveon MM small animal PET/CT. F. Rats were continuously anesthetized by 1.5% isoflurane gas mixture and fixed in a prone position under anesthesia.

minutes each time, at a temperature of about 40°C (for avoidance of burn at the needle insertion site). This was performed for 7 consecutive days at 10 days.

Principle for selecting acupoints in acupuncture and appropriate acupoints

The principle for selecting acupoints in the normal warm acupuncture group and model + warm acupuncture group was as follows. According to the classical theory in Mongolian medicine and clinical treatment, effective acupoints were selected (**Figure 1C, 1D**). Acupoint: A. Dinghui acupoint: This is the interception-point between an imaginary line joining both ears and a line from the middle of the nose to the back of head. Acupuncture at the Dinghui acupoint can treat aphonia, obnubilation, mania, visual deterioration, swirl, and headaches; B Haoyi acupoint: This is in the middle of the superior fovea of the first thoracic vertebrae. Acupuncture at the Haoyi acupoint can treat mania, palpitation, agitation, dumbness, insomnia, gray coatings on the tongue, and neck rigidity; C Xin acupoint: This is in the middle of the inferior fovea of the seventh thoracic vertebra. Acupuncture at the Xin acupoint can treat palpitations, atrial fibrillation, mania,

“Badagan and Haoyi” heart diseases, insomnia, delirium, anorexia, and delirium [10].

Sampling method

Rats in the normal group, model group, and warm acupuncture group were decollated after the experiment was finished. The prefrontal cortex, hypothalamus, and hippocampus were removed from the brains of each rat. They were weighed and preserved in a refrigerator at -70°C for later use.

PET-CT scanning experiment for rats

Rats were injected with 18F-FDG via the caudal vein (provided by the Nuclear Medicine Department of the Affiliated Hospital of Inner Mongolia Medical University, Huhhot, Inner Mongolia, China). Images of the head were acquired at 40 minutes after injection of the 18F-FDG developing agent. Each rat was placed in PET/CT (SIEMENS Inveon MM) for development (**Figure 1E, 1F**). Before PET/CT scanning, the rats were pre-anesthetized with about 3% isoflurane gas mixture (V/V, isoflurane: oxygen) and continuously anesthetized with about 1.5% isoflurane gas mixture (isoflurane: oxygen). They were fixed in a prone position under anes-

Table 1. Injection doses in the PET-CT scanning experiment for rats

No.	Injection time	Injection medicine	Dose before injection (uCi)	Residual dose in the syringe (uCi)	Actual injection dose (uCi)
1	10:15	¹⁸ F-FDG	821	35	786
2	10:43	¹⁸ F-FDG	960	30	930

thetia (See **Table 1** for injection doses and times).

CT and PET images, as well as fusion images, were obtained through the SIEMENS Inveon MM work station. The acquisition time was 10 min. The acquisition field of vision FOV was 12.7 cm. All images acquired should be subject to attenuation correction. SIEMENS Inveon MM IRW image fusion software was used to compute and analyze PET/CT cross-sectional images and obtain data. Analysis of the central response characteristics of insomnia rats in Mongolian medical warm acupuncture was conducted as follows. Software SPM2 was used for GLM-based statistical analysis. A total of 20 continuous activated pixels were defined as the activated region. Acupuncture versus insomnia rat PET images were compared to identify changes in cerebral glucose metabolisms of the insomnia rats following acupuncture. Talairach coordinate graphs and Mni-Space utility software were used for positioning in the neurotomy of the encephalic region with metabolic changes.

Detection of expression levels of NDRG2 with Western blotting

Brain tissue samples were collected from the blank control group, insomnia model group, and warm acupuncture group, respectively. The histocytes were pyrolyzed. Cytoplasm and nuclear protein supernatant were extracted. The wetting transfer method was used after it was subjected to electrophoresis for 2 hours at a constant voltage of 120 V. It was electrically transferred to polyvinylidene fluoride (PVDF) membranes and blocked in the blocking buffer containing 5% skim milk TBST. Blocked PVDF membranes were placed in the primary antibody (rabbit anti-NDRG2 polyclonal antibody; 1:200; Boster Biological Engineering, Co., Ltd., Wuhan, Hubei, China) solution diluted with Tris-HCl-buffered saline and Tween (TBST), then slowly agitated at 4°C overnight. Membranes were washed at room temperature with Western wash buffer (Beyotime Institute of

Biotechnology, Beijing, China). PVDF membranes were then incubated with secondary antibodies (fluorescein isothiocyanate-conjugated donkey anti-rabbit; 1:200; Jackson Immuno Research

Laboratories, Inc., West Grove, PA, USA) diluted with TBST (1:10,000) for 2 hours at room temperature. Enhanced chemiluminescence X-ray imaging (Beyotime Institute of Biotechnology, Beijing, China) was used to detect signals. Signal intensity was subjected to relative quantitative analysis using imaging analysis software (Image-Pro Plus version 6; Media Cybernetics, Rockville, MD, USA). Gel analysis was expressed with integrated optical density values. All reagents for Western blotting were purchased from Sigma-Aldrich (Temecula, CA, USA).

Determination of differential expression profiles of microRNAs in the brain tissues of rats before and after Mongolian medical warm acupuncture

Analysis of expression profile of microRNAs: Brain tissue samples were collected from the model rats with insomnia and rats treated with warm acupuncture. Total RNA was extracted with TRIzol Reagent. Next, miRNAs chip was used to analyze expression profiles of miRNAs in the two groups of cells. The miRNAs in samples were collected and hybridized complementary to the specific chip. Fluorescence intensity of the miRNAs 3' terminal or the marked fluorophore in the sample could be scanned following hybridization, elution, and nonspecific treatment. The miRNAs with significant differential expression could be screened after massive data were processed. According to the significance of changes in expression of miRNAs, potential target miRNAs were screened. The miRNAs quantitative detection kit for differential expression of miRNAs was used to detect differentially expressed miRNAs by means of stem-loop real-time quantification RT-PCR method.

Bioinformatic prediction and verification of target genes of differentially expressed miRNAs

Bioinformatics prediction of miRNAs used the on-line estimation software. Three major types

of software used included: TargetScan, PicTar, and miRanda. Newly-discovered eukaryon expression vector of differential miRNAs was established. The bioinformatic method was employed to predict and verify whether target molecules of miRNAs included NDRG2.

Moreover, miR-181a mimics were synthesized by the Ribobio Biotechnology Company (Guangzhou, Guangdong, China). Sequences were as follows. 5'-TCGCGCGCGCTAGAGATGGTAACGTTGTCTAT-3'; internal reference U6-sRNA: 5'-CACCACGTTTATACGCCGGTG-3'.

Real-time fluorescent quantitative PCR detection of expression levels of miR-181a in brain tissues of rats treated with warm acupuncture

For reserve transcription of miRNAs, 1 µg of the small RNA was diluted to 10 µl. Next, 2 µl of the RT primer working solution was added and 4.5 µl of the DEPC water was added. It was denatured for 5 minutes at 72°C, then cooled quickly on ice. The following reverse transcription components were added (the above mixture; 5× buffer 5 µl; 10 mM dNTP 2.5 µl; RNase inhibitor 0.5 µl; ReverTra Ace 1.0 µl). The reaction was performed for 1 hour at 42°C and 5 minutes at 95°C. It was cooled on ice and preserved.

Expression of miR-181a in brain tissues of rats treated with warm acupuncture, model rats with insomnia, and normal rats was amplified with SYBR ExTaq Mix quantification PCR. Additionally, U6 was amplified and served as internal reference control. The reaction system comprised SYBR ExTaq Mix II 12.5 µl (Ribobio, Guangzhou, Guangdong, China), upstream primer 1 µl, downstream primer µl, cDNA template 2 µl, and dH₂O 8.5 µl. Reaction conditions were as follows: 95°C, 10 seconds; 95°C, 5 seconds; 58°C, 30 seconds; Reading the plate; 72°C, 30 seconds; Reading the plate; 30 cycles in total; 72°C, 10 minutes; 55°C, 5 minutes; Dissolution curve 55°C-95°C, 0.3°C/reading the plate for 1 seconds. With U6 as the internal reference gene, the formula $2^{-\Delta\Delta Ct}$ was used to compute relative expression levels of target genes.

Culture of the neurocytes of adult rats

The hippocampus was separated under a microscope to remove the meninx and blood

vessels. They were washed three times with D-Hank's solution, cut into pieces, and digested in CD-Hank'S-dispase-DNase-papin (DDDP) (Beyotime, Beijing, China) solution at 37°C. DMEM/F12 containing 10% fetal bovine serum (FBS) was added to terminate digestion. It was centrifuged for 5 minutes at 800 revolutions/min and the supernatant was discarded. The cell sediment was re-suspended with DMEM/F12 containing 10% FBS and inoculated into a culture flask containing potyomithine (10 µg/ml) and laminin (5 µg/ml). The solution was changed to the serum-free cell medium DMEM/F12 at 24 hours. EGF and bFGF (stem cell culture medium, SCM) contained 1% N2, 2% B27, and 20 ng/mL. Cells were digested, passaged with Accutase (Beyotime, Beijing, China), and inoculated into a new culture flask as per 1:3 when cell fusion reached 80-90%. HPCs were digested into a single-cell suspension and inoculated into a culture plate or culture dish coated with polyomithine (50 µg/ml) and laminin (10 µg/ml). It was changed to a differential medium (DMEM/F12, 1 ng/mlbFGF, 1% FBS and 100 nmol/L retinoic acid (RA) (Beyotime, Beijing, China) at 24 hours after cell adherence for a higher survival rate. The solution was changed every two days. Cells were differentiated into neuronal cells after being induced for 14 days [11].

Plasmid transfection

The above neuronal cells were inoculated into a 24-well microplate with 5×10^4 cells/well. All DNA was transfected with calcium phosphate at 24 hours. The recipient expression vector and scramble and miR-181a mimic expression plasmids of the luciferase reporter gene were transfected into the cells.

Experiment on dual-luciferase reporter genes

Online estimation software TargetScan, PicTar, and miRanda were used for bioinformatic prediction of miRNA target genes. Results suggest that miR-181a and NDRG2 genes have a targeted regulation relationship (**Figure 3B**). The luciferase reporter gene plasmid containing NDRG2 3'UTR (ABI Company, CA, USA) was established. The miR-181a gene eukaryotic expression plasmid and luciferase reporter gene plasmid containing 3'UTR were used to co-transfect HEK293 cells. A luciferase dual

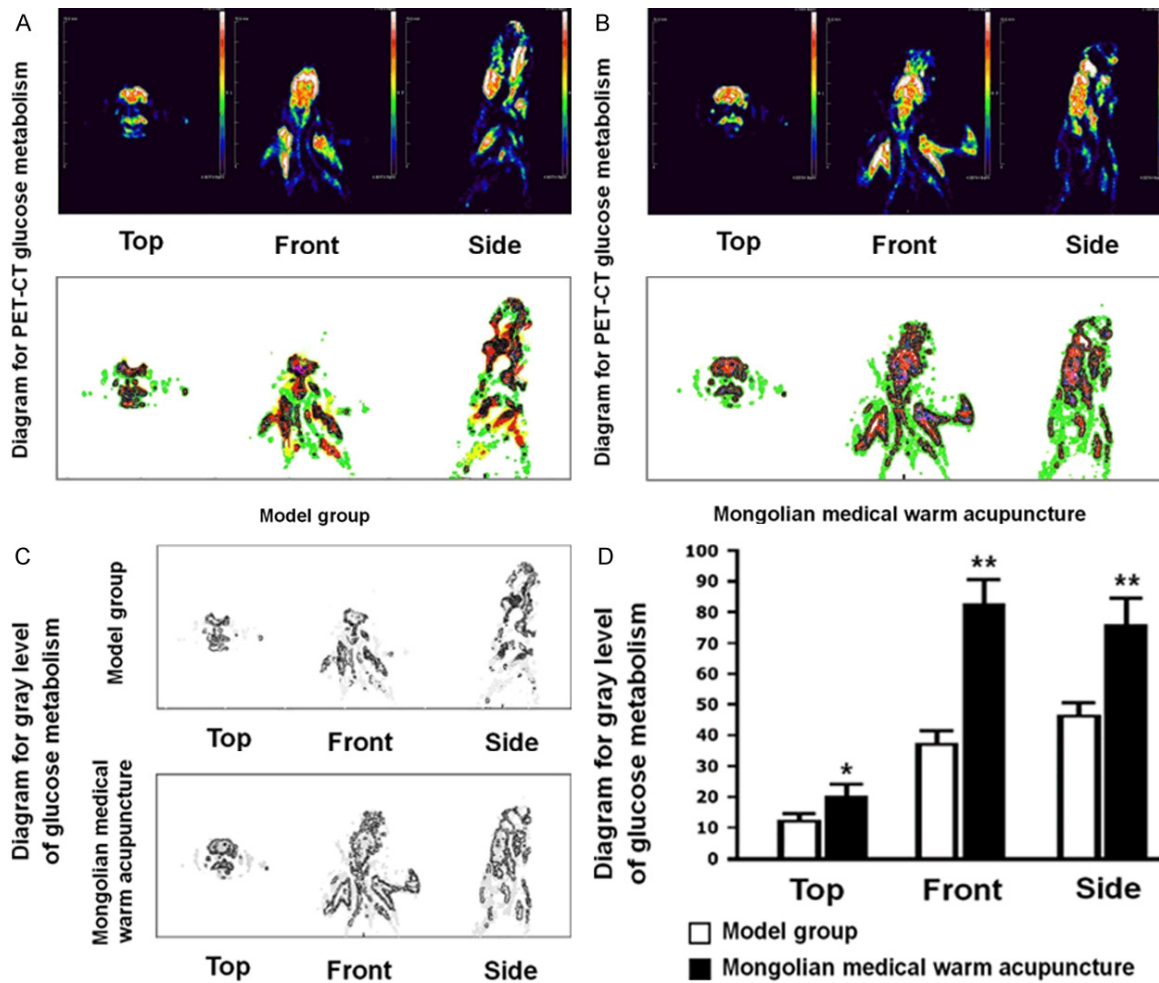


Figure 2. Small animal PET/CT was used to analyze images of glucose metabolism at the top, in the front, and on the side of the brain of the insomnia rats. A, B. The glucose metabolism at the top, in the front, and on the side of the brain of each rat in the Mongolian medical warm acupuncture group was strengthened, compared with that in the model group. C. Gray level analysis software was used to measure and analyze the images of glucose metabolism at the top, in the front, and on the side of the brain. D. Glucose metabolism at the top, in the front, and on the side of the brain of each rat in the Mongolian medical warm acupuncture group was strengthened, compared with that in the model group, $P < 0.05$. Glucose metabolism in the front and on the side of the brain was significantly strengthened, $P < 0.01$.

reporter gene experiment was conducted to identify the target relationship between miR-181a and NDRG2. Luciferase report reporter vector pMIR-REPORTTM was purchased from the ABI company (Waltham, MA, USA).

Immunohistochemical detection

Three rats were taken from each experimental group and anesthetized with 10% chloral hydrate. Aortic perfusion was performed via the left ventricle for fixation. A total of 250 mL of normal saline was used to perform pre-perfusion for removal of blood. Next, 200 mL of 4% paraformaldehyde was used for perfusion and

fixation. The whole brain tissue was separated and immersed in the same fixation solution for 4 hours. Serial frozen coronal sections were created and 2 sections were taken from each rat. Five different fields of vision within the cerebral cortex were randomly selected. The count of positive cells under each field of vision was calculated. The average value of the count of positive cells of the two sections under 10 fields of vision served as the count of positive cells of each rat. Cells were subjected to NDRG2 staining and DAB color development. Neurons of the positive cells were dark brown. Ten sections were selected for each rat that met the requirement for position. Positive neurons of

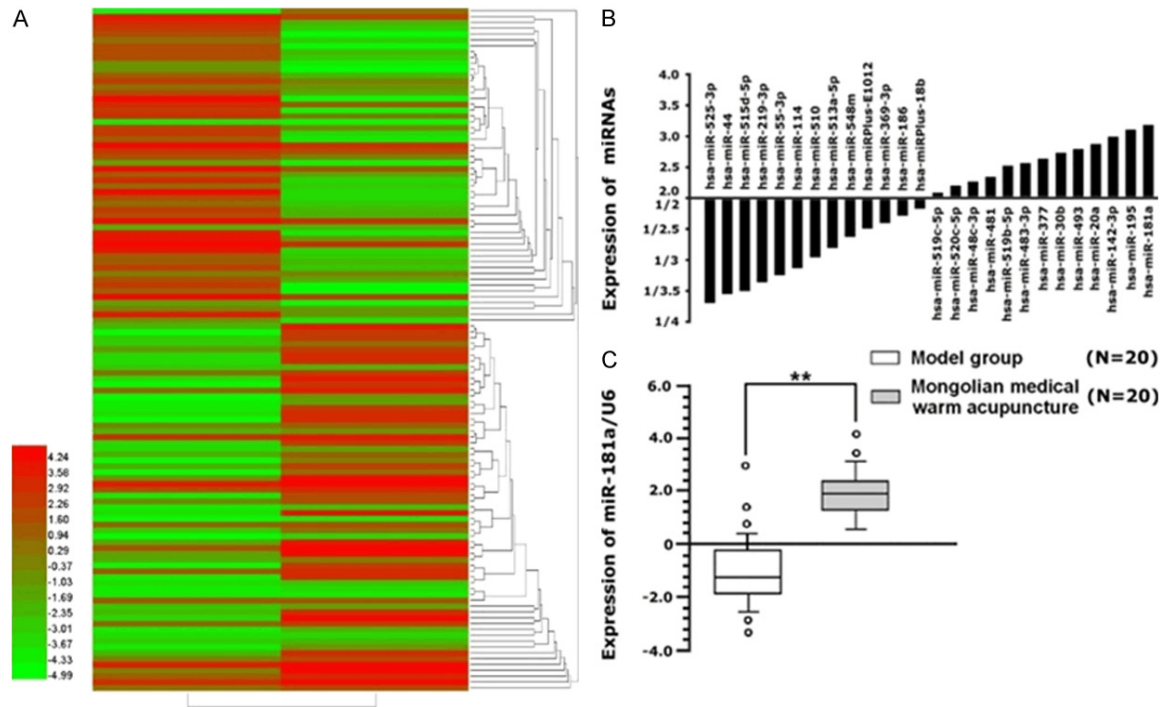


Figure 3. Difference in expression of miRNAs in the brain tissue of the rats before and after Mongolian medical warm acupuncture. A. Chip data analysis found that differences occurred in expression of 156 miRNAs in the rats subjected to Mongolian medical warm acupuncture, compared with that in the normal rats. B. Changes in expression of miR-181a were analyzed. C. Fluorescent real-time quantification validated on the level of miR-181a in 20 insomnia rats treated with warm acupuncture. The median increased to 1.9 times from 1.3 times in the model group (20) ($P<0.01$).

the NDRG2 immune response in the encephalic region were counted under a 200X phase-contrast microscope. The mean was calculated.

Detection of relevant cytokines, neurotransmitters, and their recipients

Enzyme linked immunosorbent assay (BioSino, Beijing, China) was used to detect levels of interleukins, IL-1, IL-2, and IL-6 and tumor necrosis factors - α (TNF- α) at the above site. Detection of monoamine neurotransmitters in the brain tissue: High-performance liquid chromatography and electrochemical methods (HPLC-ECD) were used to determine levels of glutamic acid (Glu) and γ -aminobutyric acid (GABA).

Statistical methods

SAM and TIGR Multiple Array Viewer software package (TMeV version 4.0) were used for unsupervised clustering analysis of chip expression profiles of miRNAs. In fluorescent real-time quantification PCR detection, software Sequence Detection system (SDS) 2.3 was used for data analysis. Expression levels of miRNAs

are expressed with Δ Ct values (Ct miRNA-Ct U6). Biological experimental data are expressed with mean \pm standard deviation (mean \pm SD). Differences between the two groups were subjected to Student's t test. Continuous data from multiple groups were analyzed using one-way ANOVA, with Tukey's post hoc test. Data of various groups were compared with Chi-squared test or Fisher's Exact Test. Differences are statistically significant when $P<0.05$. Statistical analysis used software SPSS 18.0.

Results

Glucose metabolism within the brains of the insomnia rats

Small animal PET/CT was used for image analysis of the glucose metabolism at the top, in the front, and on the side of the brains of insomnia rats. Data showed that glucose metabolism in the Mongolian medical warm acupuncture group was strengthened, compared with that in the model group (Figure 2A, 2B). Notably, gray level analysis further indicated that glucose metabolism at the top, in the front,

Table 2. Glucose metabolism in regions of insomnia rats

Brain region	Left/right	Talairach coordinate			t value	Trend	Brodmann subregion
		X	Y	Z			
Medial frontal	L	-24	38	-12	-2.56	↓	9/10
Middle frontal	R	24	-13	58	-4.02	↓	6/9
Middle temporal	L	-57	-26	-9	-3.35	↓	21
Precuneus	R	12	-51	32	-2.99	↓	30/39
ACC	L	-12	48	-4	-3.65	↓	32
MCC	R	8	-56	29	-3.74	↓	31
PCC	L	-2	-38	24	-4.21	↓	24/31
Insula	L	-44	-9	13	-4.11	↓	13
Postcentral gyrus	L	-61	-22	32	-4.68	↓	2
Fusiform	R	22	-82	-14	-3.42	↓	18/19
Hippocampus	L	-28	-22	-7	-3.68	↓	
Para-hippocampus	R	-30	-49	-4	-3.49	↓	19
Cerebellum	R	6	-68	-8	-3.95	↓	

Table 3. Changes in regions with decreased glucose metabolism in the brains of each insomnia rat

brain region	left/right	Talairach coordinate			t value	compared with the model group	Brodmann sub-region
		X	Y	Z			
Medial Frontal	L	-24	38	-12	-1.67	↑	9/10
Middle Frontal	R	24	-13	58	-1.22	↑	6/9
Middle Temporal	L	-57	-26	-9	4.35	↑	21
Precuneus	R	12	-51	32	-2.99	-	30/39
ACC	L	-12	48	-4	-3.66	↓	32
MCC	R	8	-56	29	3.67	↑	31
PCC	L	-2	-38	24	1.30	↑	24/31
Insula	L	-44	-9	13	-2.01	↑	13
Postcentral Gyrus	L	-61	-22	32	-3.21	↑	2
Fusiform	R	22	-82	-14	0.25	↑	18/19
Hippocampus	L	-28	-22	-7	-3.68	-	
Para-hippocampus	R	-30	-49	-4	-3.77	↓	19
Cerebellum	R	6	-68	-8	-1.25	↑	

and on the side of rats in the Mongolian medical warm acupuncture group was strengthened, compared with that in the model group ($P<0.05$) (**Figure 2C, 2D**). Glucose metabolism of the medial frontal, middle frontal, middle temporal, precuneus, ACC, MCC, PCC, insula, postcentral, gyrus, fusiform, hippocampus, para-hippocampus, and cerebellum in insomnia rats was decreased, compared with that in the normal rats (**Table 2**). Glucose metabolism of ACC and the para-hippocampus still decreased following Mongolian medical warm acupuncture. No changes occurred in the precuneus and hippocampus. In contrast, glucose metabolism in the medial frontal, middle frontal, middle temporal, MCC, PCC, insula, post-

central, gyrus, fusiform, and cerebellum increased (**Table 3**). The above results show significant differences in the effects of model rats with insomnia and Mongolian medical warm acupuncture on the cerebral functional activity. Mongolian medical warm acupuncture could activate most regions inhibited in the brains of each insomnia rat.

Differences in expression of miR-181a in the brain tissue of insomnia rats before and after Mongolian medical warm acupuncture

Chip data analysis found that expression of 156 miRNAs in rats treated with Mongolian medical warm acupuncture was obviously

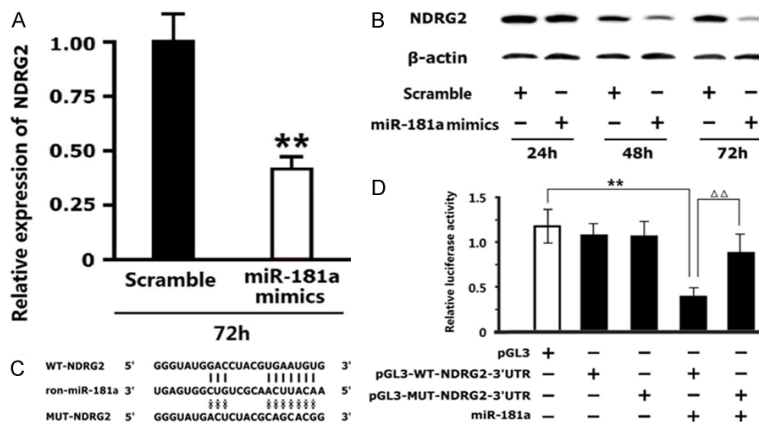


Figure 4. miR-181a inhibited expression of the NDRG2 protein. A, B. Western blotting result indicated that expression of the NDRG2 protein was inhibited and significantly down-regulated at 72 hours after addition of miR-181a mimics in the scramble group during the process where the neuronal cells of the model rats with insomnia were cultured in vitro ($P<0.01$). C. TargetScan, PicTar, and miRanda were used for bioinformatic prediction of the miRNA target gene. D. Luciferase reporter gene assay by using 293T cells indicated that no significant changes occurred in pGL3M-MUT-NDRG2-3'UTR and pGL3M-WT-NDRG2-3'UTR in the negative control group compared with pGL3M in the vacant plasmid group. No significant changes occurred in the activity in the MUT group while the fluorescence intensity in the WT group decreased significantly after transfection with miR-181a mimics.

altered (Figure 3A). Levels of miR-181a were increased by 3.2 times, compared with those in the model group (Figure 3B). This study conducted fluorescent real-time quantification PCR research on expression levels of miR-181a in 20 rats treated with warm acupuncture. It was found that the median was increased to 1.9 folds ($P<0.01$), compared to -1.3 folds in the model group (Figure 3C).

Roles of miR-181a in inhibiting expression of NDRG2 proteins

Western blotting results indicate that expression of NDRG2 in neuronal cells of model rats with insomnia was significantly downregulated at 72 hours after the addition of miR-181a mimics, compared to that in the scramble group ($P<0.01$) (Figure 4A, 4B). TargetScan, PicTar, and miRanda were used for bioinformatics prediction of miRNA target genes. Based on the prediction, miR-181a and NDRG2 might have a target regulation relationship. Wildtype and mutant plasmids containing the luciferase reporter gene of NDRG2 3'UTR were established (Figure 4C). The reporting result of luciferase in 293T cells indicated no significant changes in the MUT-NDRG2-3'UTR group,

while fluorescence intensity in the WT group was decreased significantly following addition of miR-181a mimics (Figure 4D).

Immunohistochemical detection of NDRG2 proteins

The count of NDRG2-positive neurons in the whole brain increased significantly in the insomnia model group, compared with that in the blank control group ($P<0.01$). The right-side frontal cortex, corpus striatum in the right-side caudate nucleus region, and the hippocampus were selected for observation. NDRG2 was positive in neuron plasma and occasionally present in glial cells, endothelial cells of the cerebral small vessels, and smooth muscle cells of the blood vessel wall, as well

as the epithelial cells of the choroid plexus in the third ventricle of cerebrum and endothelial cells of the ventricle wall surrounded by necrotic tissue. Positive results were also observed in neutrophils around the blood vessels. Volume shrinkage and rod-like fusion occurred in the NDRG2-positive cells of rats following administration of warm acupuncture. NDRG2 was lowly expressed in these cells, particularly in the neuron cytoplasm (Figure 5).

Detection results of IL-1, IL-2, IL-6, and TNF- α

Enzyme linked immunosorbent assay was used to detect levels of interleukins of IL-1, IL-2, and IL-6 and tumor necrosis factor- α (TNF- α). Results are shown in Figure 6A-D. Levels of interleukins of IL-1, IL-2, and IL-6, and levels of TNF- α in the hypothalamus, hippocampus, and brain prefrontal cortex of insomnia rats decreased significantly, compared with those in the blank control group. Differences were significant ($P<0.05$). Levels of IL-1, IL-2, IL-6, and TNF- α in the warm acupuncture group increased significantly, compared with those in the model group ($P<0.05$). Results indicate that warm acupuncture is significantly efficacious in treating rats with insomnia.

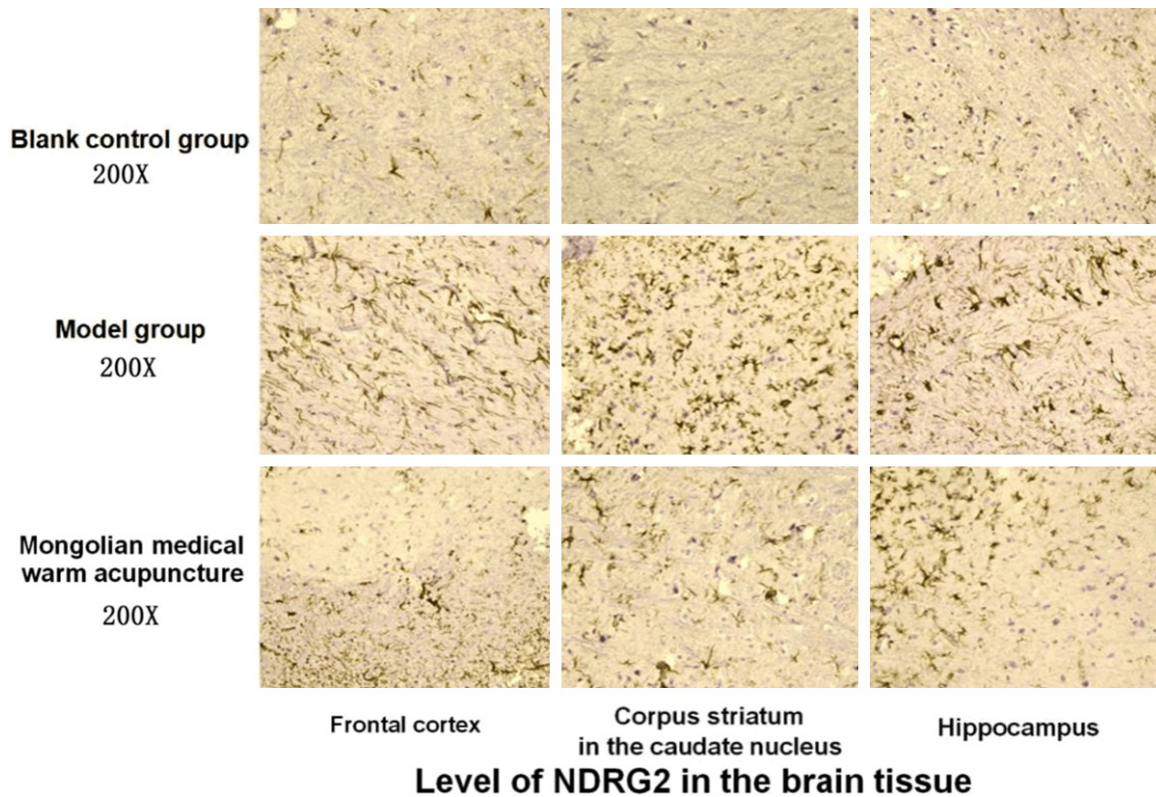


Figure 5. Immunohistochemistry diagram for Mongolian medical warm acupuncture in regulating the NDRG2 protein. The images showed the right frontal cortex, right caudate nucleus corpus striatum, and the hippocampus. Expression of NDRG2 in the cerebral cortex region in the insomnia model group increased, compared with that in the blank control group. Brown colored granules were present in the vicinity of the cell membrane of the cytoplasm of the NDRG2-positive cells. Positive cells were distributed unevenly. The positive cells were distributed densely in the cortical area. Expression could be found in various sections of brain tissue. Positive expression of NDRG2 in the frontal cortex, caudate nucleus corpus striatum, and the hippocampus decreased in the rats treated with Mongolian medical warm acupuncture. 200X amplification was performed.

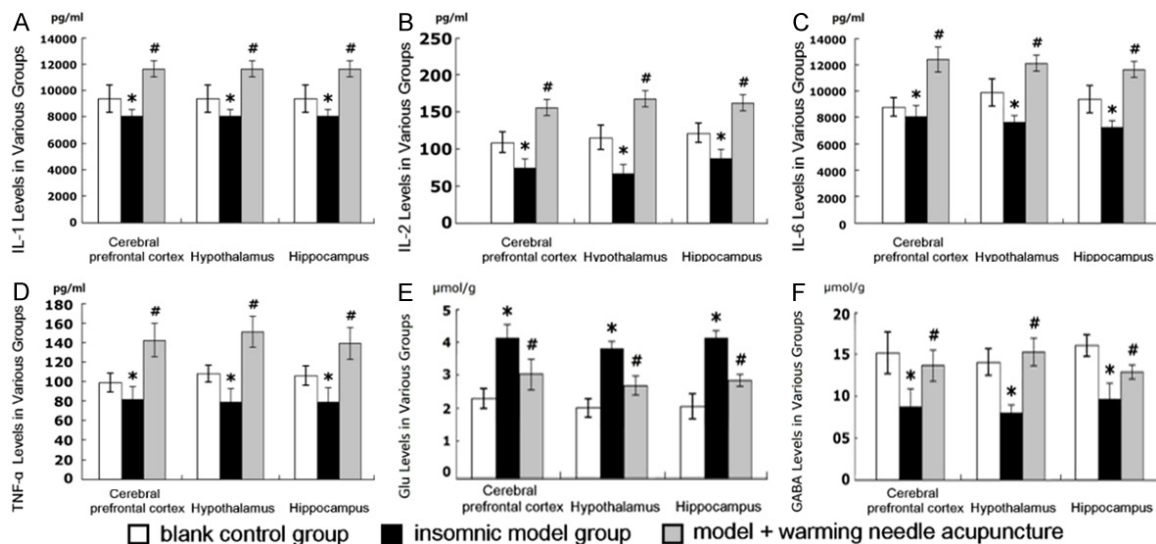


Figure 6. Levels of IL-1, IL-2, IL-6, TNF-α, and Glu, and GABA in the hypothalamus, hippocampus, and prefrontal cortex tissue of the rats. *, P<0.05, insomnia model group vs. blank control group. #, P<0.05, model + warming needle acupuncture group vs. the insomnia model group.

Detection results of Glu and GABA

Detection results of Glu and GABA in the hypothalamus, hippocampus, and prefrontal cortex tissue are shown in **Figure 6E, 6F**. Of note, levels of Glu in the insomnia model group were significantly higher than those in the blank control group ($P<0.05$). Levels of Glu were decreased significantly in the insomnia model group following warm acupuncture ($P<0.05$). Levels of GABA of rats in the insomnia model group were significantly lower than those in the blank control group ($P<0.05$). However, warm acupuncture significantly increased levels of GABA, compared with the insomnia model group ($P<0.05$).

Discussion

Mongolian medical warm acupuncture is a type of traditional external therapy for prevention and treatment of diseases, as well as rehabilitation by means of acupuncture and warm moxibustion at fixed acupoints with special silver needles. Acupuncture effects, warm effects, and specific stimulation at various acupoints interact mutually to contribute to certain biological effects for treatment of diseases [12]. A great deal of clinical research has indicated that Mongolian medical warm acupuncture is simple, efficacious, and safe in the treatment of insomnia [13]. Mongolian medicine improves sleep and treats insomnia by alleviating Haoyi, dredging Haoyi blood, and balancing Sangen [14]. In the theory of Mongolian medicine, it is believed that warm acupuncture can dredge channels, regulate qi, blood, and somatostatin, and strengthen immunity [15]. Previous findings have indicated that Mongolian medical warm acupuncture is used to stimulate the Dinghui acupoint, Haoyi acupoint, and Qianding acupoint for insomnia treatment. Thus, it is clear that Mongolian medical warm acupuncture plays a significant role in the treatment of insomnia [16].

PET-CT is an examination method combining a high-performance PET scanner and high-performance CT scanner. It marks elements of some compounds participating in human metabolism. These compounds become stable after being injected into the human body and participate in cell metabolism [17]. Molecular imaging displays and measures cell molecules in the biological process. It can analyze the biological system without disturbing the biological

system. Imaging may be performed after quantification of the molecular alteration related to diseases [18]. The spatial resolution of PET-CT can be up to millimeters and directly detects changes in glucose in the brain, reflecting the activity of the neurons. Glucose is the major source of brain cell energy. It is degraded into 6-phosphoric acid glucose in the brain. Carbon dioxide and water are generated via aerobic metabolism pathways. Additionally, Adenosine Triphosphate (ATP) is generated to provide energy for activity of the neurons. Therefore, the metabolism rate of glucose in the brain can refer to brain function [19]. Present research demonstrates that Mongolian medical warm acupuncture effectively accelerated glucose metabolism in the brain and activated the neurons of the rats for the first time.

The current experiment found that expression levels of miR-181a in the Mongolian medical warm acupuncture group increased relatively, compared with those in the model group of insomnia rats. The crystal structure of NDRG2 showed that members in the gene family lack the signal sequence for subcellular positioning and a hydrophobic region occurs at the N-terminal, suggesting that NDRG2 may have a transmembrane region [20, 21]. Based on bioinformatics analysis of the NDRG2 promoter region, it was found that NDRG2 has multiple transcription factor binding sites primarily involving tissue-specific genes, genes related to cell growth and development, and regulation of expression of stress response genes [22, 23]. NDRG2 can inhibit genes related to glucose uptake and present cell specificity, consistent with current results [24]. Mongolian medical warm acupuncture can regulate glucose metabolism in the brain by regulating expression levels of miR-181a and influencing expression of NDRG2. Modern medical research on insomnia has revealed that sleep-awakening is a physiological process involving coordination and integration of multiple systems, as well as central nerves. It represents a complicated regulation mechanism primarily associated with sleep-activated cells in the preoptic region of the brain stem reticular system, histaminergic neurons in the nipple nodule region, special nervous structures, such as cerebral cortex, neurotransmitters related to sleep-awakening, such as γ -aminobutyric acid, sleep regulation roles of the interleukin 1 and tumor necrosis factor, and regulatory mechanisms of non-peptide substances on sleep [25, 26]. The current

study also demonstrated that treatment of insomnia with Mongolian medical warm acupuncture is associated with the neurotransmitters mentioned above.

Mongolian medical warm acupuncture regulates the mechanisms and treats diseases by integrating acupuncture effects, warm effects, and specific stimulation of the acupoints via a multi-system, multi-channel, and multi-path complicated mechanism involving blood circulation, nervous system, and immunologic functions. Present data demonstrates that Mongolian medical warm acupuncture induced miR-181-NDRG2 pathways, providing biological evidence for modernization development of traditional ethnic medicine.

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Disclosure of conflict of interest

None.

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