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Original Article

Chrono-moxibustion adjusts circadian rhythm of CLOCK and BMAL1 in adjuvant-induced arthritic rats

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Abstract: Objective: The clinical symptoms of rheumatoid arthritis (RA) have significant circadian rhythms, with morning stiffness and joint pain. Moxibustion is effective in the treatment of RA, while the underlying therapeutic mechanisms remain limited. Thus, we explored whether moxibustion could adjust the circadian rhythm of RA by modulating the core clock genes CLOCK and BMAL1 at the molecular level. Methods: 144 Sprague Dawley rats were randomly divided into four groups: control group (group A), model group (group B), 7-9 am moxibustion treatment group (group C), and 5-7 pm moxibustion treatment group (group D). Each group was divided into 6 time points (0 am, 4 am, 8 am, 12 N, 6 pm, and 8 pm) with an equal number of rats at each time point. Except for group A, all rats were injected with Freund's Complete Adjuvant (FCA) 0.15 ml on the right foot pad to establish the RA model. The rats of the two moxibustion treatment groups were respectively subjected to moxibustion at 7-9 am and 5-7 pm. After 3 weeks of treatment, the tissues were collected at 6 time points during the next 24 hours. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to test the mRNA expression of CLOCK and BMAL1 in the hypothalamus and synovial tissues. CLOCK and BMAL1 protein expression in synovial tissues were detected with western blot. Results: Compared to group A, group B showed significantly down-regulated expression levels of CLOCK and BMLA1 at synovial tissue ($P < 0.05$), while no statistically significant difference was found in the hypothalamus ($P > 0.05$). The expression levels of CLOCK and BMLA1 were up-regulated in the moxibustion treatment groups in different tissues, especially in synovial tissue ($P < 0.05$) compared to group B. Nevertheless, no difference was observed between groups C and D ($P > 0.05$). Conclusions: Moxibustion could treat RA by modulating clock core genes CLOCK and BMAL1 to regulate the circadian rhythm. However, there was no significant difference between the 7-9 am moxibustion treatment group and the 5-7 pm moxibustion treatment group. This study provides a basis for research on moxibustion in the treatment of RA.

Keywords: Rheumatoid arthritis, chrono-moxibustion, clock gene, CLOCK, BMAL1, circadian rhythm

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease presenting with circadian variations of clinical features, such as morning stiffness and joint pain. RA represents one of the most prevalent chronic inflammatory diseases, affecting about 1% of the world population [1]. Although the etiology of RA is not fully elucidated, previous studies have demonstrated several risk factors related to the development of RA, including shift work and endocrine system disorder [2, 3]. RA has typical circadian oscillations, and this clinical characteristic has not been taken into account in current treatment regimens [4]. Therefore, we provide a novel insight into the treatment of RA by focusing on the diurnal variations of clinical features in RA.

Circadian rhythms are endogenous self-sustaining oscillations with periods of 24 hours, controlled by a series of positive and negative feedback loops of clock genes. Disruption of circadian rhythms is involved in a variety of diseases, especially RA [5]. Several studies have identified that the diurnal rhythm of RA symptoms was closely linked to variations in pro-inflammatory cytokines in the late night and

early morning, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α [6, 7]. A study found that changes in the circadian oscillation of cytokines in RA patients might be adjusted by the level of clock gene expression [8]. The disturbed circadian clocks have an essential impact on the development of RA [9]. The biological clock rhythmically regulates the expression levels of various genes as well as being influenced by clock genes [10]. Thus, the pathologic changes of RA are adjusted by core clock genes, and the biological clock may play a key role in the treatment of RA by regulating the disrupted diurnal rhythm.

As a traditional Chinese therapy, moxibustion has been widely used in the treatment of RA [11]. Previous studies have shown that moxibustion could significantly alleviate pain, swelling, and stiffness in the early morning for RA patients [12]. Besides, moxibustion could adjust the expression level of core circadian clock genes such as BMAL1, CLOCK, PER, and CRY [13]. Our previous research has demonstrated that moxibustion regulated the circadian rhythm of REV-ERBα in RA rats without side effects to effectively treat RA [14]. Furthermore, some breakthrough progress has been made in the treatment of collagen-induced arthritis rats with drug chronotherapy [15]. Long-term nocturnal administration of small doses of glucocorticoids extended-release could consistently improve RA patients' clinical symptoms and effectively control disease progression [16]. Consequently, we hypothesized that moxibustion might treat RA by modulating the expression levels and circadian rhythms of CLOCK and BMAL1 in different tissues of adjunctive RA rats, and chrono-moxibustion could produce different efficacy differences in the treatment of RA.

To verify this hypothesis, we used qRT-PCR and western blot to detect the expression quantity of CLOCK and BMAL1 in the hypothalamus and synovial tissue. In the study, the adjuvant arthritis (AA) rat model was used to investigate the treatment efficacy of moxibustion in RA. Then the data were analyzed using SPSS 17.0 and Halberg Cosiner software. In conclusion, we explored the molecular biologic mechanism of moxibustion in the treatment of RA, to provide a reference for the clinical treatment of RA.

Materials and methods

Experimental animals

144 Sprague-Dawley rats were supplied by Chengdu Dashuo Laboratory Animal Co., Ltd. (Chengdu, China), half male and half female. All rats were acclimatized for one week. Rats were housed in a pathogen-free environment (12 h light/dark cycle, 20-25°C, 55±5% relative humidity). Rats had free access to food and water. All animal procedures were approved by the Institutional Ethics Committee of South Western Medical University (No. 2018030973).

Experimental design

After adaptive feeding for one week, rats were randomly divided into four groups (each group n = 36): control group (group A), model group (group B), 7-9 am moxibustion treatment group (group C), and 5-7 pm moxibustion treatment group (group D). Except for group A, all rats were subcutaneously injected with 0.15 ml FCA (Sigma) at the right foot pad to establish the AA rat model. Meanwhile, rats in group A received the same volume of physiological saline. Significant swelling of the foot indicated successful modeling compared to group A.

Moxibustion therapy

Moxibustion treatment was given one week after FCA injection for moxibustion treatment groups (**Figure 1A**). Both groups C and D were performed on ST36 and BL23 at 7-9 am and 5-7 pm, respectively (**Figure 1B**). The moxibustion treatments were received for a consecutive three weeks (a total of 18 times). Rats in other groups were fixed in the same way but untreated with moxibustion.

Measurement of foot volume

The right paw volume of each rat was periodically measured on days 0 d, 7 d, 14 d, 21 d, and 28 d of the experiment by using a self-made foot volume measuring device [14]. To reduce errors, each rat was measured three times. To reduce the bias caused by subjective visual readings, each operator was fixed.

Foot swelling index

Foot swelling degree calculation formula in rats: foot swelling degree after modeling = (foot

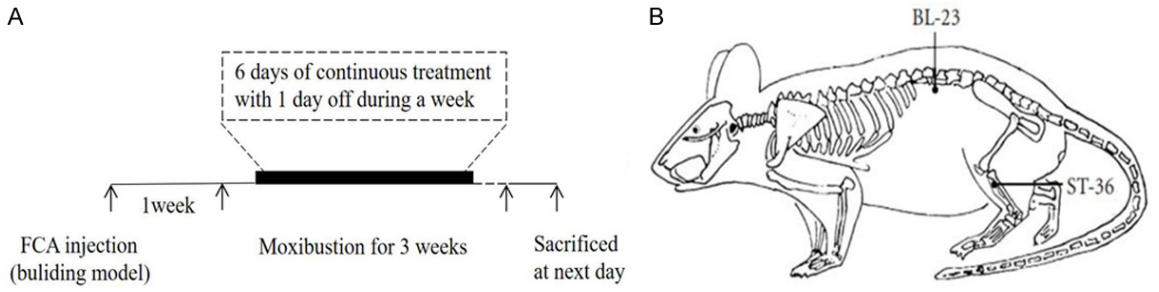


Figure 1. Experimental illustration. A. Time line of experiment. B. Schematic diagram showing that rats received moxibustion at Zusanli (ST 36) and Shenshu (BL23).

volume after modeling-foot volume before modeling)/foot volume before modeling; foot swelling degree after treatment = (foot volume after treatment-foot volume before modeling)/foot volume before modeling.

Sample collection

Rats were sacrificed at the end of the treatment (on the 29 d of the experiment). Samples were collected at the corresponding times, such as 0 am, 4 am, 8 am, 12 N, 6 pm, and 8 pm.

Quantitative real-time PCR

The total RNA was extracted from hypothalamus and synovial tissue using Tri reagent (Sigma, USA) according to the manufacturer's protocol. mRNA was reverse transcribed into cDNA using the High-Capacity cDNA Synthesis kit (Invitrogen, USA). The relative mRNA levels of CLOCK and BMAL1 were measured with a SYBR-green detection system (Applied Biosystems, Foster City, CA, USA). β-actin was used as a reference gene for normalization of different transcript values. According to gene sequences in the NCBI database, specific primers were designed by the Primer Premier 5.0 software (Premier Biosoft, USA). cDNA was amplified with the following primers: for CLOCK, 5'-AAGATGACAAGGACAAAGCAA-3' and 5'-TGCGTAAAAAATCAATGCTCT-3'; for BMAL1, 5'-CAGAAGCAAACTACAAGCCAA-3' and 5'-GGTCACATCCTACGACAAACA-3'; for β-actin, 5'-CGAGTACAACCTTCTTGCAGC-3' and 5'-ACCCATACCCACCATCACAC-3'. The relative mRNA expression levels were evaluated with the $2^{-\Delta\Delta c_T}$ method. All PCR assays were performed in triplicate.

Western blotting

The protein expression of CLOCK and BMAL1 was detected by western blot analysis. According to the manufacturer's recommendations, total protein was extracted using the KC™ Cell and Tissue Total Protein Extraction kit (KC-415; KangChen Bio-tech Inc., Shanghai, China). The protein concentration was examined using the KC™ bicinchoninic acid assay protein quantification kit (KC-430; KangChen Bio-tech Inc.). Total protein was resolved by SDS polyacrylamide gelelectrophoresis (SDS-PAGE), and transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS) for 2 h at room temperature, and then incubated with primary antibodies for BMAL1 (1:1000, Santa, USA), CLOCK (1:1000, Santa, USA) and GAPHD (1:5000, KangChen Bio-tech Inc., China) overnight at 4°C. Following washing in TBST for 6 times, the membrane was incubated with anti-mouse secondary antibody (1:5,000, KangChen Bio-tech Inc., China) at room temperature for 1 h. After 6 times of washing with TBST, immunoreactive bands were detected using the KC™ chemiluminescence kit (KC-420, KangChen Bio-tech Inc.). Finally, the protein bands were scanned (Tanon 4600 SF; Tanon Science and Technology Co., Ltd., Shanghai, China) and quantified using Image J Version 1.8.0 (National Institutes of Health, Bethesda, USA). Levels of GAPDH were used for standardization.

Statistical analysis

All data were presented as the means ± standard deviation (SD). All statistical analyses were performed by SPSS 17.0 statistical software. The Student's t-test was used for the comparison between two groups, while the

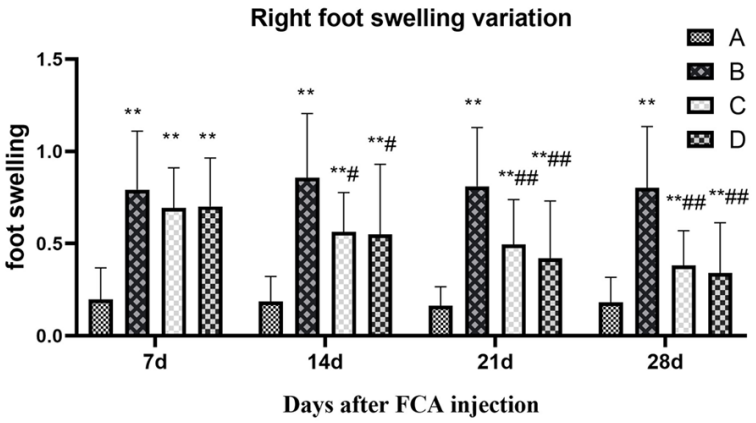


Figure 2. Effect of moxibustion on right foot swelling. Rats were randomly divided into four groups: control group (A), model group (B), 7-9 am moxibustion treatment group (C) and 5-7 pm moxibustion treatment group (D). Group differences were analyzed by one-way ANOVA. *P < 0.05, **P < 0.01 vs. control, #P < 0.05, ##P < 0.01 vs. model, n = 36 for each group.

one-way analysis of variance (ANOVA) test was used to analyze the differences among multiple groups. The Halberg cosiner software was used to analyze the circadian rhythm (Departments of Biology, School of Chemistry and Life Science, Suzhou University of Science and Technology, Suzhou, Jiangsu, China) [17]. P < 0.05 was considered to indicate a significant difference.

Results

Moxibustion relieved the right foot swelling symptom of RA rats

Firstly, moxibustion was investigated to determine whether it could relieve swelling in the right foot of RA rats. Compared to group A, the other three groups had right foot volumes that were significantly increased (P < 0.01) on the 7th day (after modeling), which indicated that the building adjuvant RA model was successful (**Figure 2**). Compared to group B, there was no significant difference between groups C and D (P > 0.05), indicating that the three groups were comparable at baseline after moulding.

The swelling of the right foot in group B was still higher (P < 0.01) compared to group A on the 14th day (after 1 week of moxibustion treatment), while the swelling of the right foot was significantly decreased in groups C and D (P < 0.05) compared to group B. There was a decreased tendency for foot swelling symptoms in group D compared to group C, but the difference was not significant (P > 0.05).

On the 21st day (after 2 weeks of moxibustion treatment) and 28th day (after 3 weeks of moxibustion treatment), compared to group A, the right foot of group B was still swollen (P < 0.01). The right foot swelling of rats in groups C and D was decreased (P < 0.01) compared to group B. Compared with group C, the right foot swelling of group D had a downward tendency. However, the difference between groups C and D was not significant (P > 0.05).

These results illustrated that moxibustion treatment could alleviate RA rats' foot swelling. However, there was no statistical difference between groups C and D (P > 0.05). In addition, we further investigated the effects of moxibustion on the key clock genes CLOCK and BMAL1 of RA rats at different times.

Core circadian genes are highly expressed in hypothalamus

Next, the effects of moxibustion on CLOCK and BMAL1 expression in different tissues were examined. CLOCK and BMAL1 mRNA expression in the hypothalamus and synovial tissue were detected by qPCR. As shown in **Figure 3A**, **3B**, the relative expressions of CLOCK and BMAL1 were significantly higher in the hypothalamus than in the synovial tissue. The experiment indicated that CLOCK and BMAL1 were differentially expressed in various tissues of rats. In addition, the expression levels of CLOCK and BMAL1 were reduced in the hypothalamus of group B, but the difference was not significant compared to group A (P > 0.05). The results confirmed that the expression levels of biological clock genes were more stable in the central circadian clock than in the peripheral circadian clock.

The expressions of CLOCK and BMAL1 in group B were dramatically lower compared with group A, particularly in the synovial tissue (P < 0.05). Nevertheless, the CLOCK and BMAL1 expression levels in groups C and D were increased

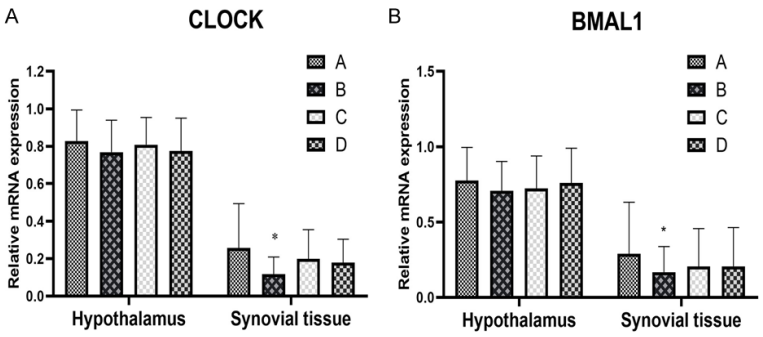


Figure 3. CLOCK and BMAL1 mRNA expression in different tissues. A. CLOCK mRNA expression in hypothalamus and synovial tissue. B. BMAL1 mRNA expression in hypothalamus and synovial tissue. *P < 0.05 vs. control, **P < 0.01 vs. control, n = 36 for each group.

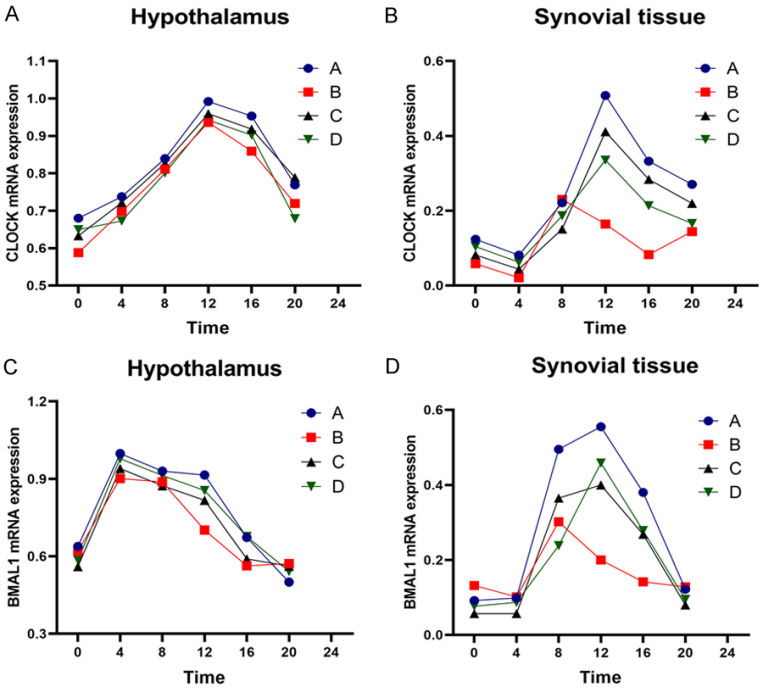


Figure 4. Relative expression mRNA of CLOCK and BMAL1 in hypothalamus and synovial tissue at different time points. A, B. Relative expression mRNA of CLOCK in different tissues at different time points (n = 36 for each group). C, D. The expression of mRNA of BMAL1 in different tissues at different time points (n = 36 for each group).

Expression of CLOCK and BMAL1 mRNA in various tissues at different time points

To further explore the effects of moxibustion on the modulation of biological clock genes in RA rats, we observed the expression of CLOCK and BMAL1 mRNA in various tissues at different time points. The mRNA expression of CLOCK and BMAL1 changed over time in the hypothalamus and synovial tissue among all groups of rats (**Figure 4**). In addition, the trends of CLOCK and BMAL1 in the hypothalamus were consistent across groups of rats. The peak time of BMAL1 expression in the hypothalamus was around 04:00 and the trough time was around 20:00, with both peak and trough times in the dark phase (**Figure 4A**). Moreover, the peaks of CLOCK expression in the hypothalamus were around 12:00 in the light phase, while the trough time was around 0:00 in the dark phase (**Figure 4C**). In synovial tissue, the peaks of CLOCK and BMAL1 expression in group B were around 8:00 in the light phase, while the peaks of mRNA expression for other groups were around 12:00 in the light phase. Furthermore, the trough time of CLOCK and BMAL1 expression was around 04:00 in the dark phase (**Figure 4B**, **4D**).

compared to group B. However, there was no significant difference in treatment effects between groups C and D (P > 0.05).

The results proved that the effect of RA on peripheral tissues was more significant, and moxibustion could markedly increase the mRNA expression of CLOCK and BMAL1 in synovial tissue. In addition, chrono-moxibustion had no obvious difference in the treatment groups.

Chrono-moxibustion regulated the core clock genes CLOCK and BMAL1 protein expression of RA rats

Clock gene expressions at the protein level were studied in the synovial tissue of RA rats. As shown in **Figure 5**, compared to group A, the expression of CLOCK protein was significantly reduced in the synovial tissue of group B (P < 0.01), and group D was reduced (P < 0.05) compared to group A. Compared to group B, the

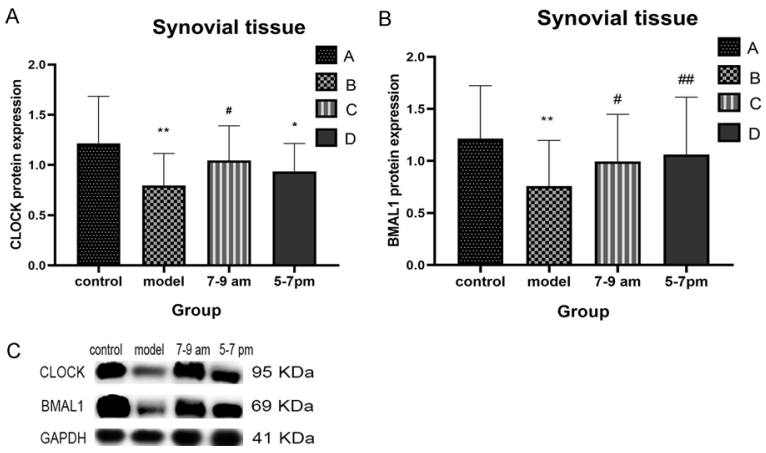


Figure 5. Protein expression of core clock gene CLOCK and BMAL1 in synovial tissue. A. CLOCK protein expression in synovial tissue. B. BMAL1 protein expression in synovial tissue. C. Western blotting analyses for CLOCK and BMAL1 in synovial tissue. *P < 0.05, **P < 0.01, vs. Control, #P < 0.05, ##P < 0.01, vs. model, n = 36 for each group.

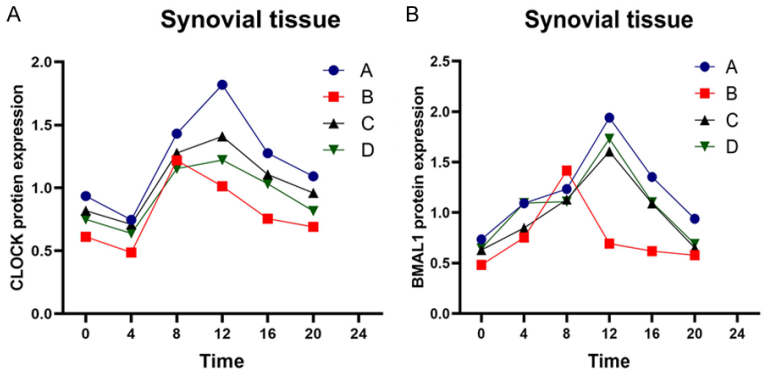


Figure 6. Protein expression of CLOCK and BMAL1 in synovial tissue at different time points. A. Protein expression of CLOCK in synovial tissue at different time points (n = 36 for each group). B. Protein expression of BMAL1 in synovial tissue at different time points (n = 36 for each group).

CLOCK protein expression of synovial tissue was significantly increased in group C (P < 0.05). Compared to group C, the CLOCK protein expression of group D was decreased. However, there was no significant difference in treatment effects between groups C and D (P > 0.05).

The expression of BMAL1 protein in the synovial tissue of group B was reduced (P < 0.05), whereas in groups C and D were nosignificant differences (P > 0.05) compared with group A. There was a significant difference in the expression of BMAL1 protein in both groups C and D (P < 0.05) compared to group B. Compared to group C, the BMAL1 protein expression of group D was increased, but the difference was not significant (P > 0.05).

The results proved again that the expression level of the core clock gene was more easily disturbed in peripheral tissues of RA rats. Moxibustion could increase the protein expression of CLOCK and BMAL1 in RA rats. Furthermore, no significant difference was observed in the chrono-moxibustion treatment groups.

Expression of CLOCK and BMAL1 protein in synovial tissue at different time points

The protein expression of CLOCK and BMAL1 in the synovial tissue of each group changed with time (**Figure 6A**, **6B**). The peak time of CLOCK and BMAL1 expression in group B of synovial tissue was around 08:00, which is about 4 hours ahead compared to other groups. In addition, the changed trend of CLOCK and BMAL1 of other groups was almost consistent, and the peak time of expression was around 12:00, which appeared in the light phase, while the trough time was around 04:00.

The circadian rhythms of CLOCK and BMAL1 mRNA expression in different tissues were altered by chrono-moxibustion treatment

We found that there were diurnal oscillations in CLOCK and BMAL1 mRNA in the hypothalamus of all groups (**Figures 7** and **8**, P < 0.05), while there were no circadian rhythms of CLOCK and BMAL1 mRNA in the synovial tissue of group B (**Figures 9B**, **10B**, P > 0.05). After receiving moxibustion, the treatment groups of RA rats showed a diurnal rhythm in synovial tissue (**Figures 9C**, **9D**, **10C**, **10D**, P < 0.05). The peak phases of the CLOCK mRNA expression in the rats' hypothalami were -195.1° (about 13:00), -187.3° (about 12:30), -197.1° (about 13:08), and -191.6° (about 12:47) in groups A, B, C, and D, respectively (**Figure 7**). The peak phase

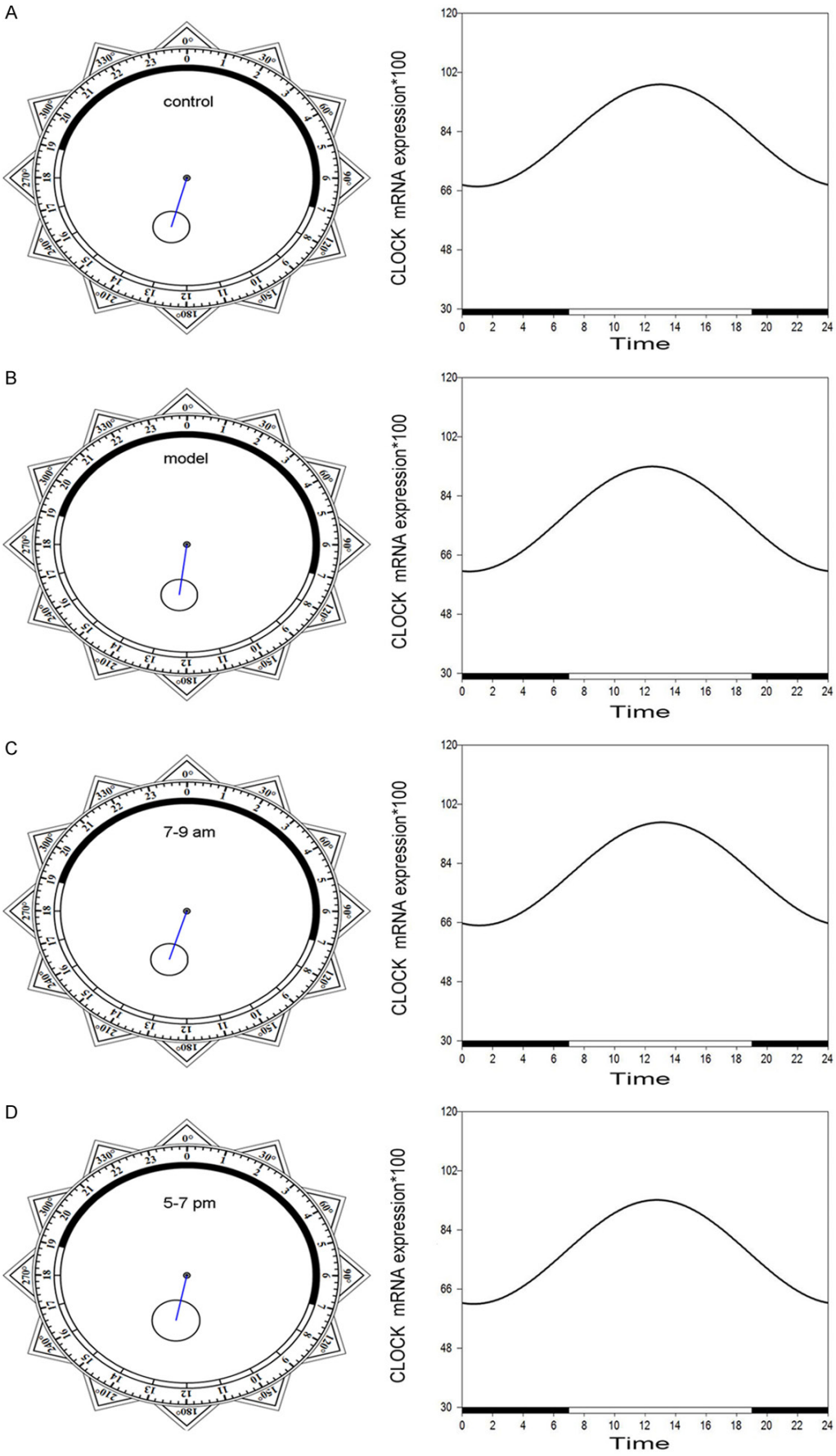
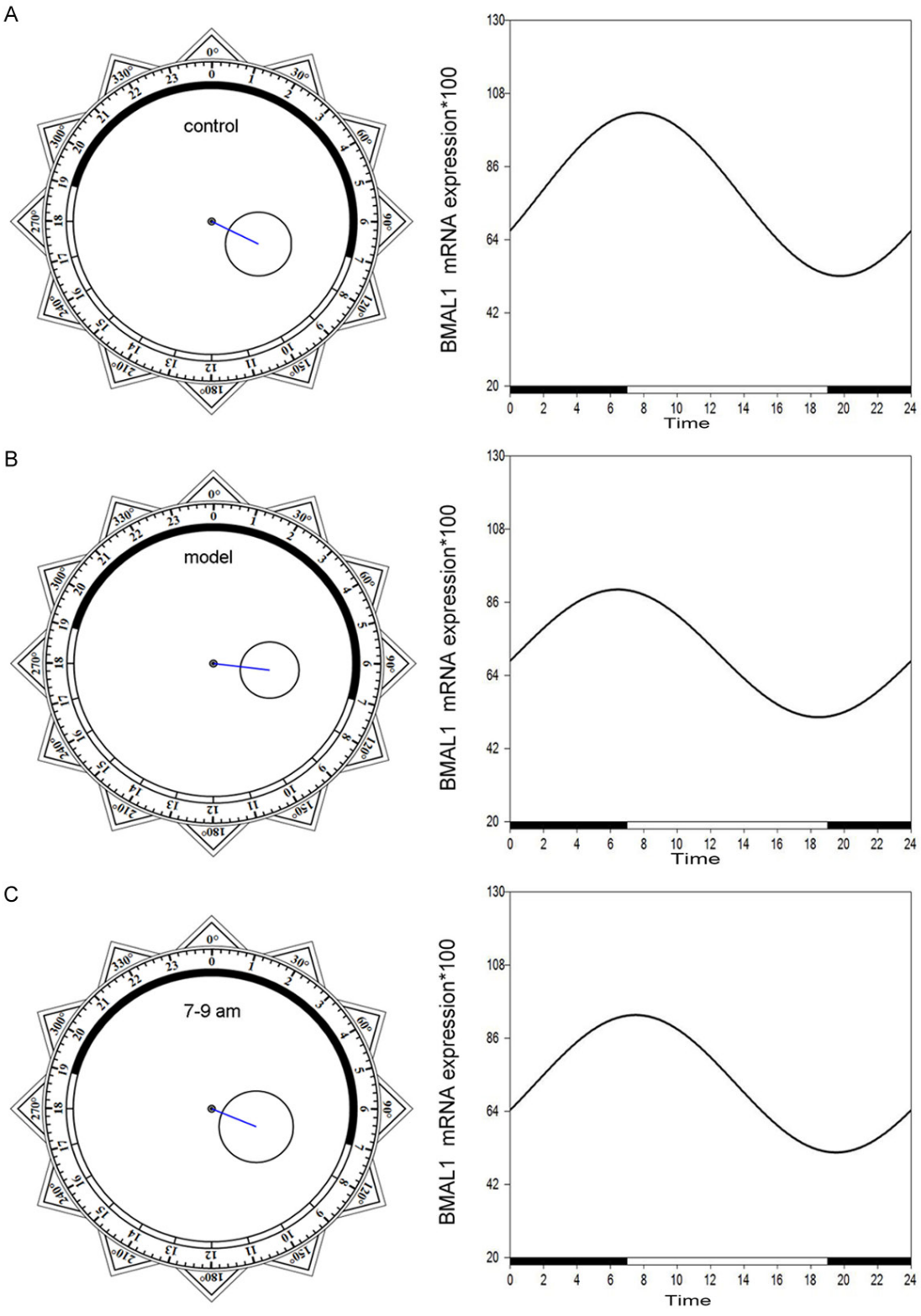

A
control
CLOCK mRNA expression*100
Time
B
model
CLOCK mRNA expression*100
Time
C
7-9 am
CLOCK mRNA expression*100
Time
D
5-7 pm
CLOCK mRNA expression*100
Time

Figure 7. The zero-amplitude test and fitting curve for circadian rhythm of CLOCK mRNA expression in the hypothalamus. A. Control group (group A). B. Model group (group B). C. 7-9 am moxibustion treatment group (group C). D. 5-7 pm moxibustion treatment group (group D). In the zero-amplitude test, the pole (zero-point) of the polar coordinate was not overlapped by the circles, suggesting that the test results have circadian rhythm ($P < 0.05$). In the fitting curve, x-axis indicates the time in the 24-hour light-dark cycle; the y-axis indicates the expression level of CLOCK mRNA in the hypothalamus. The white and dark sections on the x-axis represent the time in light and dark. 360° = 24 hours, 00:00 = 0°, n = 36 for each group.



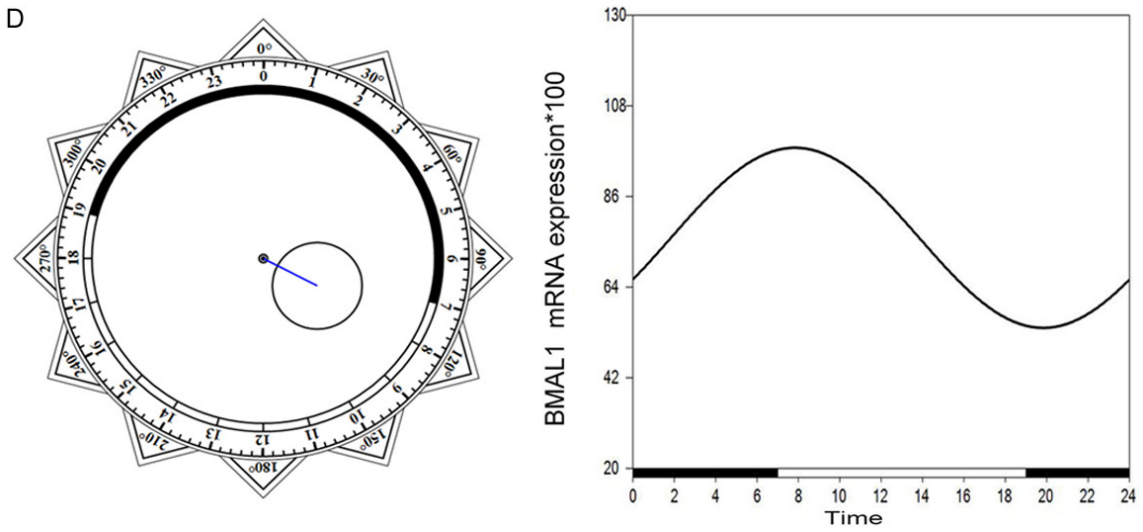


Figure 8. The zero-amplitude test and fitting curve for circadian rhythm of BMAL1 mRNA expression in hypothalamus. A. Control group (group A). B. Model group (group B). C. 7-9 am moxibustion treatment group (group C). D. 5-7 pm moxibustion treatment group (group D). In the zero-amplitude test, the pole (zero-point) of the polar coordinate was not overlapped by the circles, suggesting that the test results have circadian rhythm ($P < 0.05$). In the fitting curve, x-axis indicates the time in the 24-hour light-dark cycle; the y-axis indicates the expression level of BAML1 mRNA in the hypothalamus. The white and dark sections on the x-axis represent the time in light and dark. 360° = 24 hours, 00:00 = 0°, n = 36 for each group.

of group B was approximately 0.5 hours earlier compared with group A. Compared to group B, the peak phases of groups C and D were shifted backward and closer to group A. Compared to group C, the peak phase of group D was about 20 minutes ahead. The results proved that the peak phase of CLOCK mRNA expression that appeared in the middle of the light phase and group C was nearer to the peak phase of group A. The peak phases of BMAL1 mRNA expression in the hypothalamus (**Figure 8**) of the rats in groups A, B, C, and D were respectively located at -117.2° (about 7:49), -96.9° (about 6:28), -112.7° (about 7:31), and -118.4° (about 7:54). Compared to group A, the peak phase of group B was significantly advanced by about 1.5 hours. The peaks of groups C and D were respectively shifted backward about 1 hour and 1.5 hours compared to group B. Compared to group C, the peak phase of group D was delayed by about 20 minutes. The results showed that BMAL1 mRNA expression peaked early in the light phase and group D was closer to the peak phase of group A.

In the synovial tissue, the peak phase of CLOCK mRNA expression (**Figure 9**) was -207.9° (about 13:52) in group A, -171.6° (about 11:27) in group B, -212.9° (about 14:12) in group C, and -199.1° (about 13:16) in group D. Compared to group A, the peak phase of group B was earlier by about 2.5 hours. And the peak phase of group C and group D were respectively delayed by around 3 hours and 2 hours compared to group B. Compared to group C, the peak phase of group D was ahead by about 1 hour. The results indicated that the CLOCK mRNA expression peaked mid in the light phase and that group C was closer to the peak phase of group A. The peak phases of the BMAL1 mRNA expression (**Figure 10**) in group A, group B, group C, and group D were separately -173.8° (about 11:35), -147.3° (about 9:49), -173.3° (about 11:33), and -184.5° (about 12:18). The peak phase of group B was significantly advanced by about 1.5 hours compared to group A. Compared with group B, the peak phases of groups C and D were dramatically shifted rearward by about 2 hours. Compared to group C, the peak phase of group D was delayed by around 40 minutes. The findings demonstrated that the peak of BMAL1 mRNA expression occurred in the mid-light phase and that group C was closer to the peak phase of group A.

The experiment showed that the expression of BMAL1 and CLOCK mRNA peaked in the light

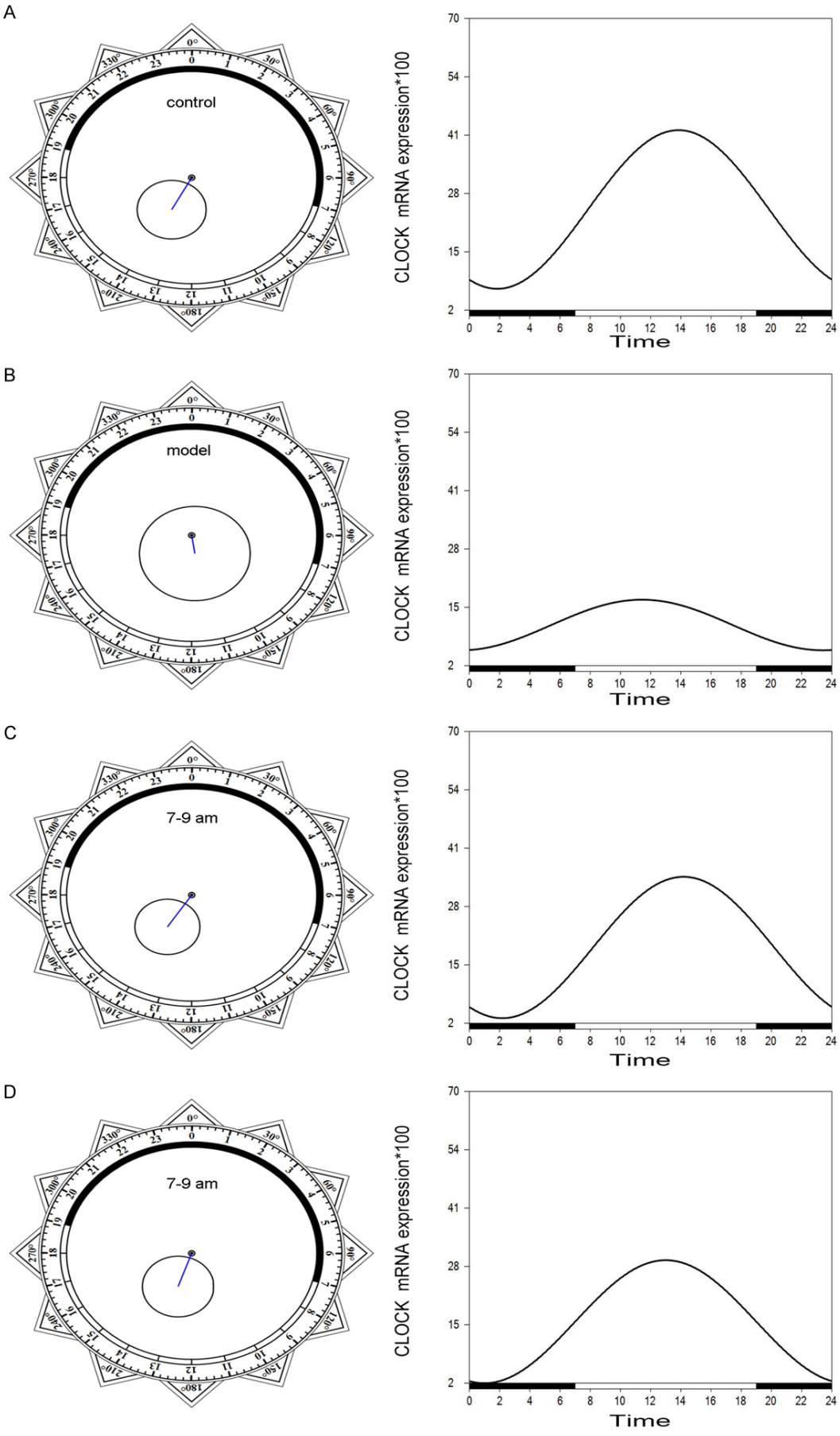

A
control
CLOCK mRNA expression*100
Time
B
model
CLOCK mRNA expression*100
Time
C
7-9 am
CLOCK mRNA expression*100
Time
D
7-9 am
CLOCK mRNA expression*100
Time

Figure 9. The zero-amplitude test and fitting curve for circadian rhythm of CLOCK mRNA expression in synovial tissue. A. Control group (group A). B. Model group (group B). C. 7-9 am moxibustion treatment group (group C). D. 5-7 pm moxibustion treatment group (group D). In the zero-amplitude test, the pole (zero-point) of the polar coordinate was not overlapped by the circles, suggesting that the test results have circadian rhythm ($P < 0.05$). In the fitting curve, x-axis indicates the time in the 24-hour light-dark cycle; the y-axis indicates the expression level of CLOCK mRNA in the synovial tissue. The white and dark sections on the x-axis represent the time in light and dark. 360° = 24 hours, 00:00 = 0°, n = 36 for each group.

phase. The peak phases of CLOCK and BMAL1 expression in group C and group D were closer to group A. The present results prove that moxibustion can regulate the mRNA expression of core clock genes to some degree and restore their disrupted circadian rhythm, thus achieving therapeutic effects. However, there was no statistically significant difference between group C and group D.

Circadian rhythm in protein expression in synovial tissue of RA rats

Some meaningful results for CLOCK and BMAL1 protein expression were analyzed in synovial tissue. The expression of circadian clock genes, CLOCK and BMAL1 protein in the synovial tissue of each group of rats showed different circadian rhythms (**Figures 11** and **12**). We found that a loss of circadian rhythm was evident in the synovial tissue of CLOCK and BMAL1 in group B ($P > 0.05$), whereas circadian oscillations were still present in other groups ($P < 0.05$). The peak phase of CLOCK protein expression (**Figure 11**) in synovial tissue was -187.5° (about 12:30) in group A, -164.8° (about 10:59) in group B, -183.6° (about 12:14) in group C, and -181.2° (about 12:05) in group D.

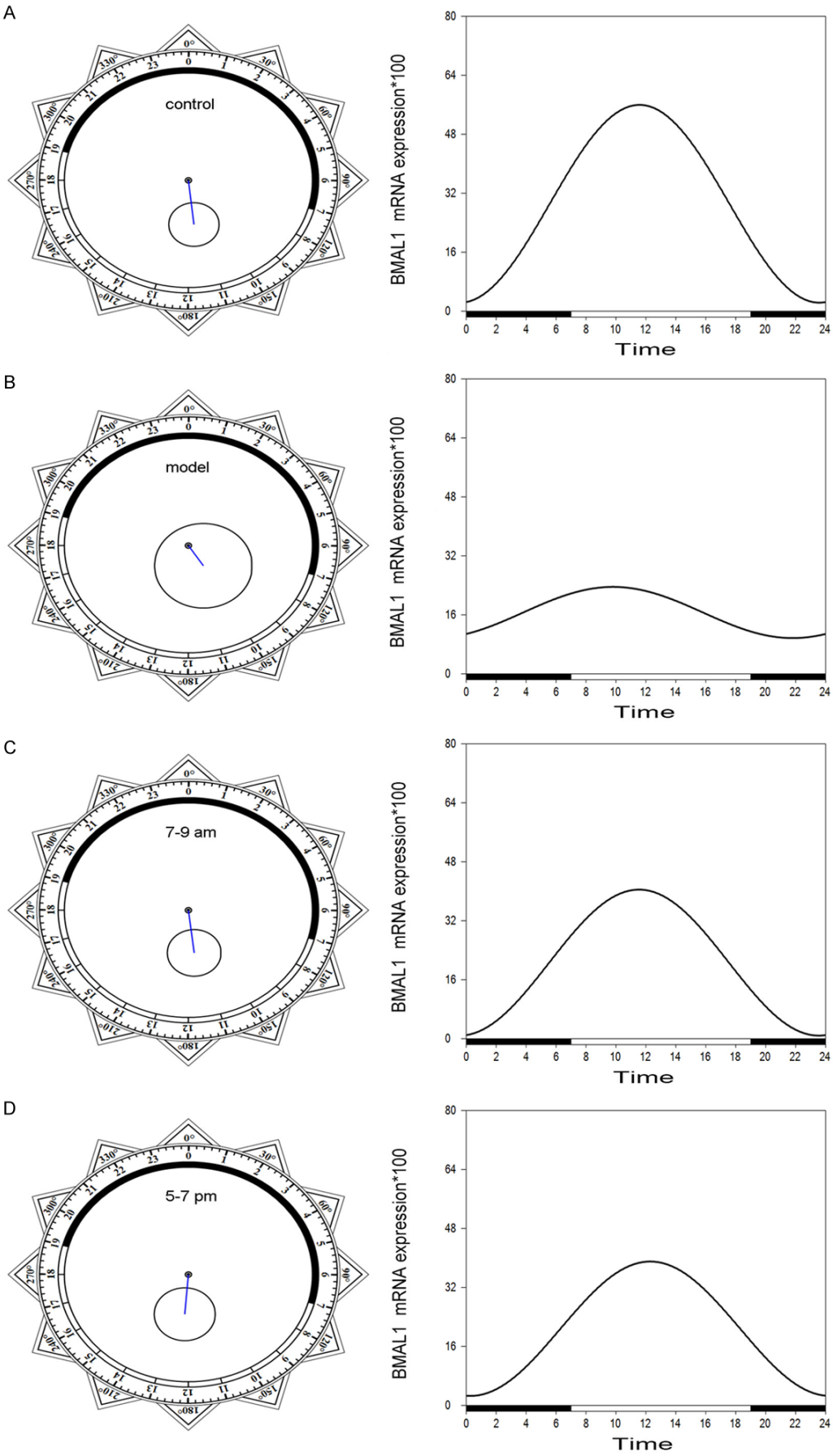
Compared to group A, the peak phase of group B was about 1.5 hours ahead. The peak phases of group C and group D were separately pushed back about 74 minutes and 65 minutes compared to group B. Compared to group C, the peak phase of group D was earlier around 9 minutes. The results indicated that the peak of CLOCK protein expression appeared in the middle of the light phase and the peak phase of group C was closer to group A. In addition, the peak phases of the BMAL1 protein expression in synovial tissue were -179.0° (about 11:56), -123.9° (about 8:16), -163.8° (about 10:55), and -171.2° (about 11:24) in group A, group B, group C, and group D, respectively (**Figure 12**). Compared to group C, the peak phase of group D was delayed by approximately 0.5 hours. The results showed that the peak of BMAL1 protein expression occurred in the middle of the light phase and the peak phase of group D was closer to group A. The results of western blotting were consistent with the PCR results in CLOCK and BMAL1 of synovial tissue.

Results again demonstrated a disturbance or disappearance of the circadian rhythm in RA rats. The treatment groups of RA rats could benignly adjust circadian oscillation after moxibustion treatment and recover their circadian rhythm. However, no difference was observed between groups C and D.

Discussion

The clinical symptoms of RA present pronounced circadian rhythms, where joint pain and stiffness are most apparent in the early morning and reduced in the afternoon [18]. Clinical studies confirmed that serum IL-6 levels in RA patients increased from 10:00 pm and peaked at 7:00 am compared to healthy controls, which positively correlated with the degree of morning stiffness and painful swollen joints in RA [19]. TNF-α concentration was abnormally elevated in serum of RA patients reaching peak levels during the early morning [20]. Due to its low cost, safety, and effectiveness, moxibustion has been recognized as a popular complementary alternative therapy for the treatment of RA patients. Modern studies have proved that moxibustion can relieve the inflammatory response in RA patients [21]. However, many relevant studies have focused on exploring the efficacy of moxibustion in the treatment of RA [22], while lacking attention to the diurnal rhythmical changes in RA. Therefore, circadian rhythmic changes in the clinical manifestations of RA should be taken into account when choosing treatment regimens for RA.

In our study, we randomly divided the rats into 4 groups: control group (group A), model group (group B), 7-9 am moxibustion treatment group (group C), and 5-7 pm moxibustion treatment group (group D). Except for group A, all rats were injected with 0.15 ml of FCA in the right


A
control
BMAL1 mRNA expression*100
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B
model
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Time
C
7-9 am
BMAL1 mRNA expression*100
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D
5-7 pm
BMAL1 mRNA expression*100
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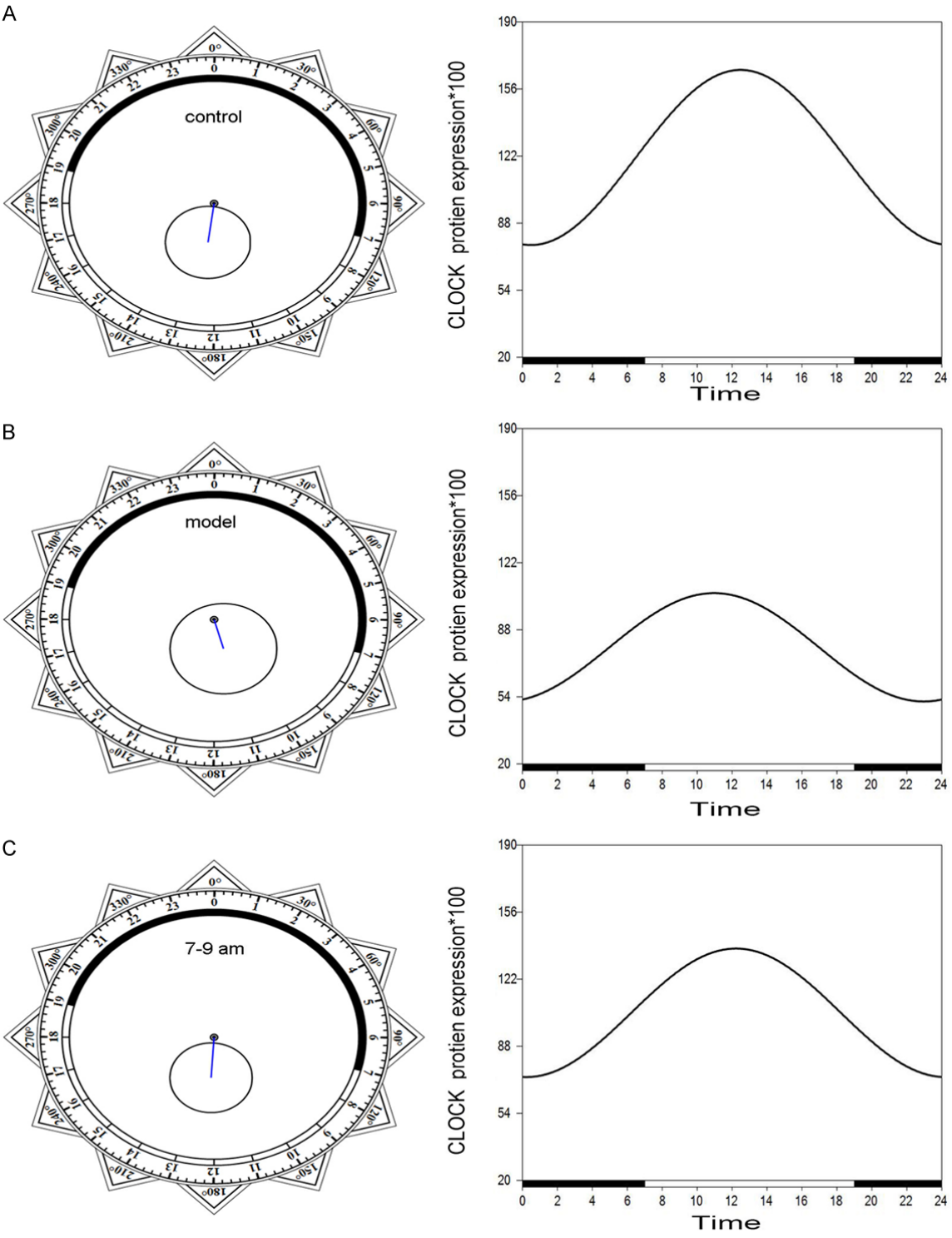
Figure 10. The zero-amplitude test and fitting curve for circadian rhythm of BMAL1 mRNA expression in synovial tissue. A. Control group (group A). B. Model group (group B). C. 7-9 am moxibustion treatment group (group C). D. 5-7 pm moxibustion treatment group (group D). In the zero-amplitude test, the pole (zero-point) of the polar coordinate was not overlapped by the circles, suggesting that the test results have circadian rhythm ($P < 0.05$). In the fitting curve, x-axis indicates the time in the 24-hour light-dark cycle; the y-axis indicates the expression level of BMAL1 mRNA in the synovial tissue. The white and dark sections on the x-axis represent the time in light and dark. 360° = 24 hours, 00:00 = 0°, n = 36 for each group.



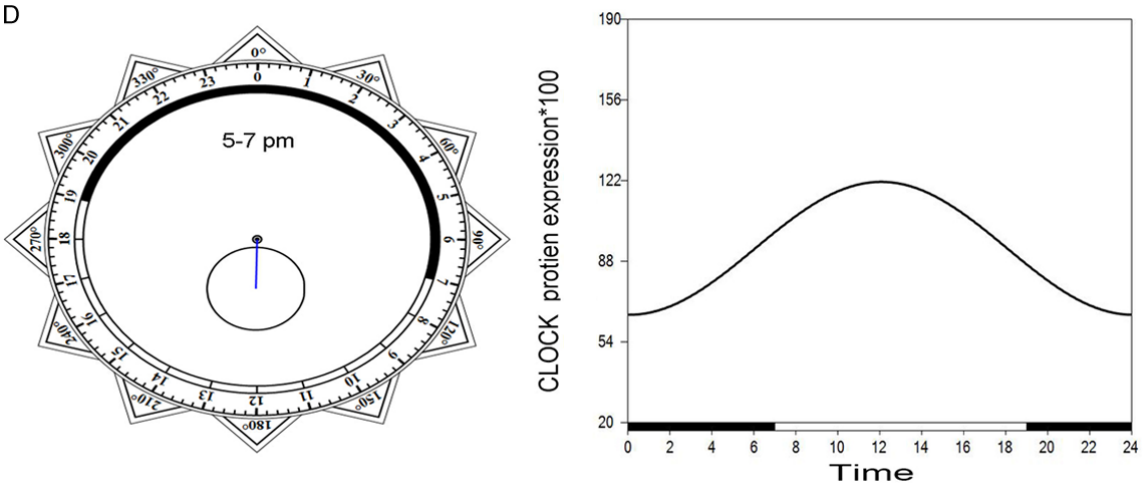
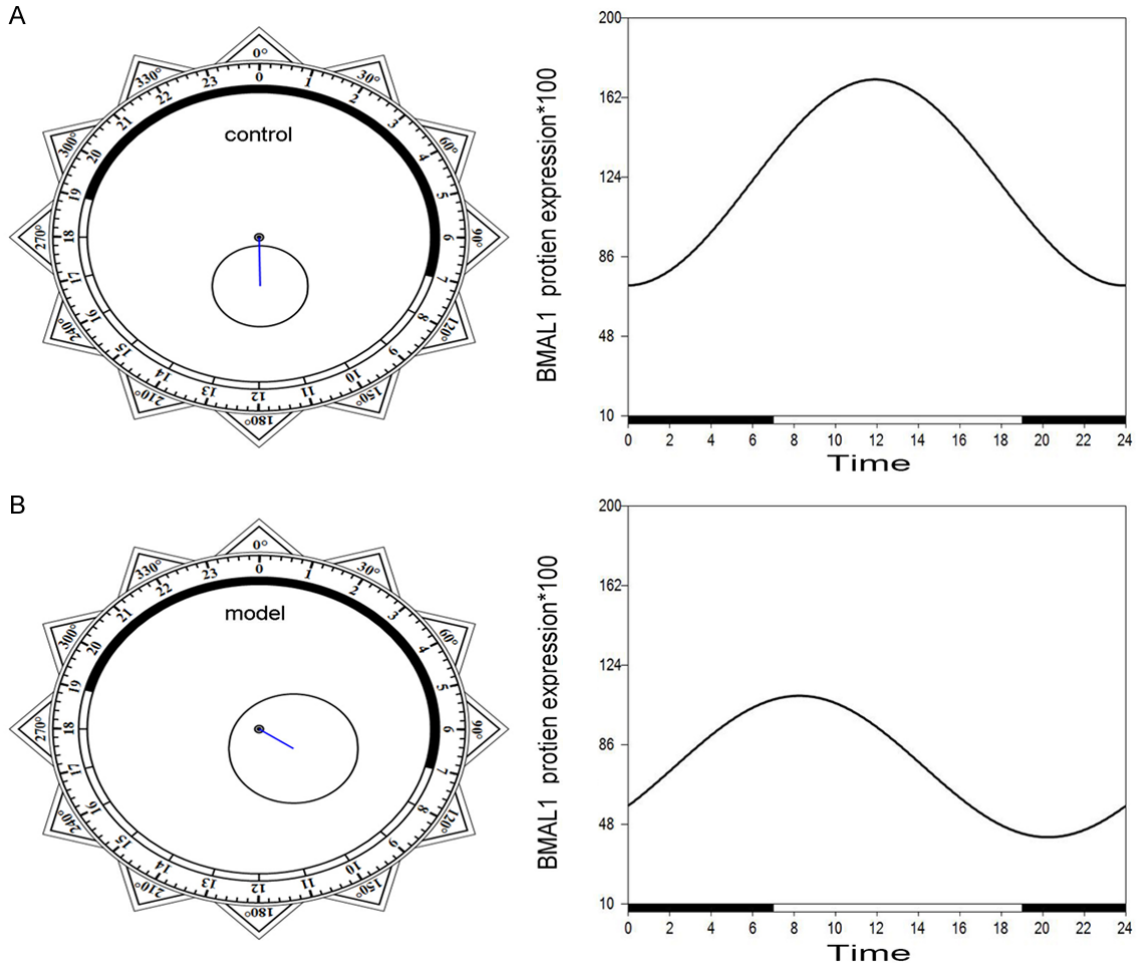


Figure 11. The zero-amplitude test and fitting curve for circadian rhythm of CLOCK protein expression in synovial tissue. A. Control group (group A). B. Model group (group B). C. 7-9 am moxibustion treatment group (group C). D. 5-7 pm moxibustion treatment group (group D). In the zero-amplitude test, the pole (zero-point) of the polar coordinate was not overlapped by the circles, suggesting that the test results have circadian rhythm ($P < 0.05$). In the fitting curve, x-axis indicates the time in the 24-hour light-dark cycle; the y-axis indicates the expression level of CLOCK protein in the synovial tissue. The white and dark sections on the x-axis represent the time in light and dark. 360° = 24 hours, 00:00 = 0°, n = 36 for each group.



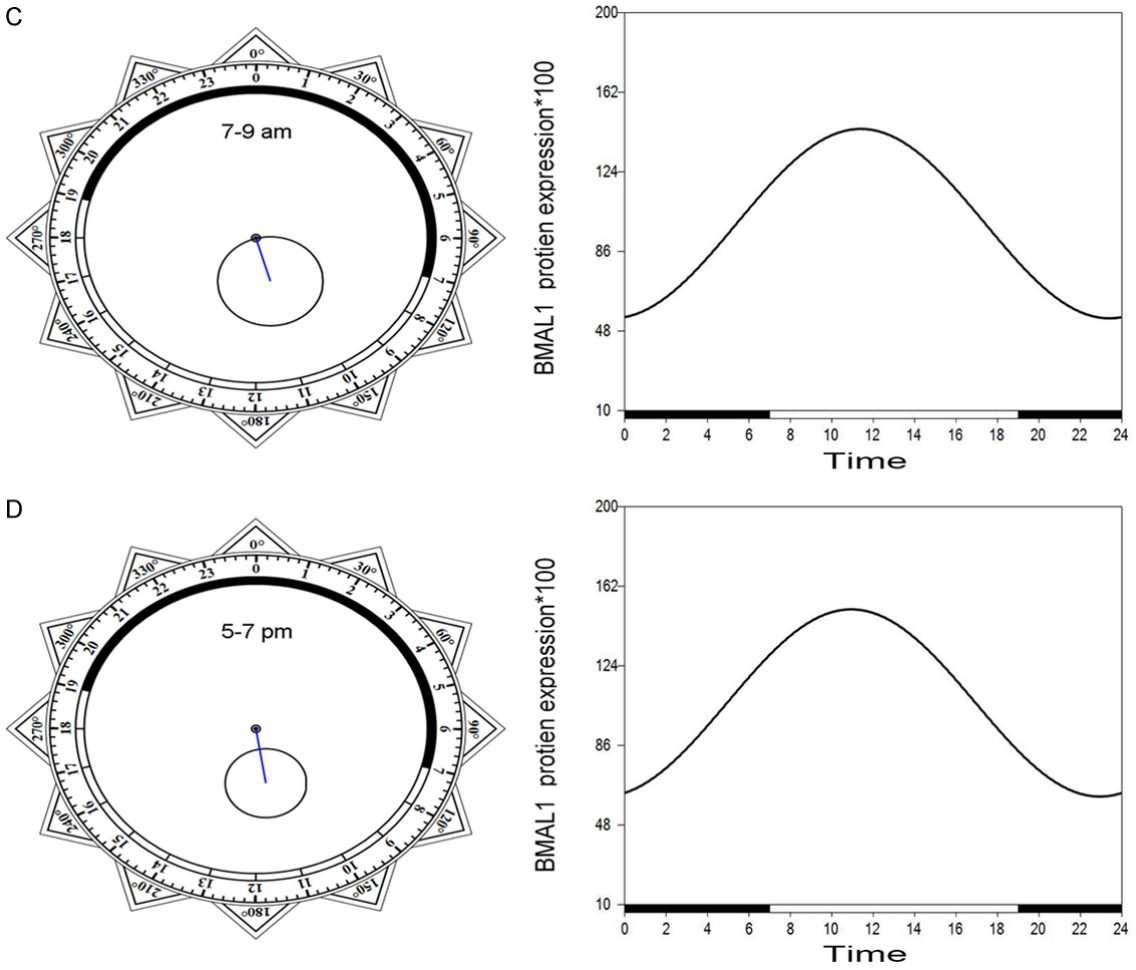


Figure 12. The zero-amplitude test and fitting curve for circadian rhythm of BMAL1 protein expression in synovial tissue. A. Control group (group A). B. Model group (group B). C. 7-9 am moxibustion treatment group (group C). D. 5-7 pm moxibustion treatment group (group D). In the zero-amplitude test, the pole (zero-point) of the polar coordinate was not overlapped by the circles, suggesting that the test results have circadian rhythm ($P < 0.05$). In the fitting curve, x-axis indicates the time in the 24-hour light-dark cycle; the y-axis indicates the expression level of BMAL1 protein in the synovial tissue. The white and dark sections on the x-axis represent the time in light and dark. 360° = 24 hours, 00:00 = 0°, n = 36 for each group.

foot pad to establish the AA rat model. The same volume of saline was also given to the group A rats. Compared to group A, significant swelling of the right foot demonstrated successful modelling. After one week of FCA injection, the two treatment groups were given continuous moxibustion treatment for three weeks. All rats were sacrificed on the day after the last moxibustion treatment, and samples were collected at the corresponding times for ZT groups. The core clock genes CLOCK and BMAL1 expression levels in the hypothalamus and synovial tissue of rats were measured by western blot and qPCR. Previous studies demonstrated that moxibustion could regulate circadian rhythms of REV-ERBα in RA rats, coupled with the fact that REV-ERBα is involved in the CLOCK/BMAL1 core feedback loop and is a transcriptional repressor of the biological clock cycle [14]. Modified-release prednisone given at bedtime could effectively treat RA [23]. Therefore, we investigated the effects of chrono-moxibustion on the expression of the core clock genes CLOCK and BMAL1 in RA rats and provided a reliable basis for the clinical treatment of RA with moxibustion at the chronotherapeutic level.

Our results showed that the degree of foot swelling in RA rats could be relieved by moxibustion treatment, indicating that moxibustion is an effective strategy for treating RA. A retro-

spective study confirmed that there were anti-inflammatory effects of moxibustion on RA rats [24]. Moxibustion was effective in the treatment of RA [25], and it suppressed the inflammatory response of RA through down-regulating the levels of pro-inflammatory cytokines TNF-α and IL-1 [26]. This agrees with earlier research demonstrating that moxibustion is a highly effective treatment for RA. In addition, we found that the biological clock genes were differentially expressed in various tissues, and the expression levels and rhythms of clock genes in the central circadian clock were more stable than in the peripheral circadian clock. The circadian clock is an intrinsic and autonomous timekeeping system that includes the central clock in the suprachiasmatic nucleus (SCN) and the peripheral clock in other tissues [27]. The SCN integrates the environmental time information to entrain its phase and then transmits this information to other oscillators in brain regions and peripheral organs [28]. The peripheral circadian clock can be regulated and influenced by the central circadian clock. The circadian rhythm of PER2 was disturbed only in peripheral organs without any effect on the central circadian clock in the SCN [29]. Therefore, diurnal rhythms are more stable in the central system than in peripheral tissues. Our qPCR results indicated that the mRNA expressions of key clock genes CLOCK and BMAL1 in the hypothalamus were relatively stable in all groups, so we did not examine the protein levels of CLOCK and BMAL1 in the hypothalamus.

In the study, the circadian rhythms of CLOCK and BMAL1 disappeared in the synovial tissue of the RA model. In contrast, after moxibustion treatment, the diurnal oscillations of the 7-9 am moxibustion treatment group and the 5-7 pm moxibustion treatment group were restored and neared to the control group. However, there was no difference between the 7-9 am moxibustion treatment group and the 5-7 pm moxibustion treatment group. Circadian rhythms exist in most cells and tissues and are regulated by the expression of clock genes. Circadian clock genes form transcriptional-translational feedback loops and generate periodic circadian oscillations [27]. There was a mutual communication between circadian rhythm and immune system involving in RA pathogenesis [30]. Disturbed circadian rhythms could lead to a variety of diseases, and the pathologic rhythms of disease in turn aggravate disturbed circadian rhythms. For example, RA patients' symptoms could be altered by the circadian clock. In turn, RA also notably disturbed the biological clock expression of patients [31]. Women who worked shifts significantly increased their risk of suffering from RA [32]. CLOCK and BMAL1, the core clock genes of the circadian clock, are involved in the pathologic progression of RA by modulating immune function to link circadian rhythms and inflammation [33, 34]. Consequently, chrono-moxibustion adjusted the expression of CLOCK and BMAL1 to treat RA.

However, there were some limitations and shortcomings in this study. Firstly, the sample size was relatively small and we explored the expression of BMLA1 and CLOCK only in the synovial tissue and hypothalamus. In the study, we found evidence that moxibustion could adjust the diurnal rhythm of key clock genes to treat RA. Nevertheless, the mechanism of moxibustion regulated the clock genes, which remains to be further investigated. Secondly, more detailed results on the relationship between clock genes and RA are unclear and further studies are needed. In order to clarify the mechanism of chrono-moxibustion for RA, more core clock genes need to be analyzed and research with larger sample sizes needs to be performed.

In summary, our study demonstrated that chrono-moxibustion could treat RA by regulating the expression of key clock genes CLOCK and BMAL1 in adjusting the circadian rhythm. The synovial tissue of CLOCK showed an increasing trend in expression levels at the 7-9 am moxibustion treatment group compared to the 5-7 pm moxibustion treatment group. In contrast, there was a tendency for the synovial tissue expression levels of BMAL1 to increase in the 5-7 pm moxibustion treatment group compared to the 7-9 am moxibustion treatment group. However, there were no significant efficacy variations between the 7-9 am moxibustion treatment group and the 5-7 pm moxibustion treatment group. Our study might give new insight for the clinical treatment of RA by moxibustion.

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Disclosure of conflict of interest

None.

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