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Review Article

Moxibustion benignantly regulates circadian rhythm of REV-ERBα in RA rats

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Abstract: The key clinical symptoms and previous findings of RA show a circadian variation, with more prominent joint swelling, stiffness, and pain occurring in the early morning. Moxibustion is able to relieve RA in various pass ways, however, there is no verifying study results for the pathological rhythm of RA. Therefore, we conducted this work to verify whether moxibustion could adjust RA circadian rhythm according to regulate core clock genes. Based on these previous findings that circadian timekeeping is disturbed in RA at molecular level, the aim of this study was to observe the influence of moxibustion on expression level and circadian rhythm of REV-ERBα at different tissues of RA rats. Furthermore, the expression level of core clock genes closely related to RA were evaluated by RT-PCR. 96 SD rats were randomly assigned as 1:1:1:1 ratio to 4 groups for normal control group, RA model group, 5-7 am moxibustion group, and 5-7 pm moxibustion group. RT-PCR was used to measure the relatively expression quantity of REV-ERBα, CLOCK, BMAL1, and PER2 in hypothalamus, hippocampus, and adrenal gland. In RA rats, the expression level of REV-ERBα mRNA were up-regulated in different tissues, and moxibustion potentially up-regulated them in different degrees. In untreated RA rats, the circadian rhythm of REV-ERBα mRNA in hippocampus and adrenal gland both disappeared ($P>0.05$) and moxibustion was able to recover them ($P<0.05$). The expression level of CLOCK and PER2 mRNA in hippocampus and adrenal gland were down-regulated significantly ($P<0.05$) in RA model rats, while moxibustion up-regulated both of them in hippocampus ($P<0.05$). These results suggested together that moxibustion can benign regulate circadian rhythm of REV-ERBα in different tissues of RA rats. It was revealed that moxibustion not only recovered the losing diurnal oscillation of REV-ERBα in hippocampus and adrenal gland, but also adjusted the circadian rhythm of REV-ERBα in hypothalamus, hippocampus, and adrenal gland to close the normal circadian pattern.

Keywords: Rheumatoid arthritis, moxibustion, core clock genes, REV-ERBα, circadian rhythm

Introduction

Rheumatoid Arthritis (RA), one of the most prevalent chronic inflammatory joint disease affecting 0.5%-1% of the population world-wild [1], is well-characterized by a circadian rhythm of clinical manifestation more serious in the early morning. Previous studies have revealed that the diurnal oscillation of RA symptoms is contributed to the early morning rise in circulating levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and TNF-α [2-4]. Furthermore, disturbed biological clock, which is controlled by circadian clock genes, plays an important impact on RA pathology, as well as intertwine with pro-inflammatory cytokines of RA [5, 6]. Consequently, this temporal variation in disease pathology is directed by core circadian clock genes, both at a systemic level, through signaling pathways derived in the central clock, and at a local level by autonomous clocks found within inflammatory organs and cells [7].

Moxibustion has been adapted to treat RA for long time and play an effective role at various pathways in RA [8]. There are documentary evidences for moxibustion benign regulates circadian rhythm of IL-1β, IL-6, and TNF-α [9, 10], as well as expression level of core circadian clock genes such as CLOCK, BMAL1, PER, and CRY [11, 12]. Then we provided a hypothesis that

moxibustion could benign the expression level and circadian rhythm of REV-ERBα in different tissues to adjust RA pathological diurnal pattern, so that rhythmically repress the inflammation of joints.

Thus, to add to the evidence in moxibustion benign regulating pathological circadian rhythm, the RT-PCR was adapted to measure the expression quantity of REV-ERBα, CLOCK, BMAL1, and PER2 in hypothalamus, hippocampus, and adrenal gland, then analysis data by SPSS21.0 for windows and Halberg Cosiner software.

Materials and methods

Animal

A total of 96 healthy adult SD rats (SCXK2015-30) with a body weight of 160-180 g were randomly allocated into 4 groups in a 1:1:1:1 ratio as normal control group, RA model group, 5-7 am moxibustion group, and 5-7 pm moxibustion group. There were 24 rats in each group and 6 rats in each Zeitgeber Time group (0 am, 6 am, 12 N, 6 pm). Mice were housed on a 12:12 h light-dark cycle (lights on 6 am, lights off on 6 pm) during whole process of experiment. After 14 days adaptation, except for control group, all rats were injected subcutaneously FCA at right foot pad to establish AA animal model. 1 week later, both moxibustion treating groups received wheat-moxibustion treatment on ST36 and BL23 at 5-7 am and 5-7 pm respectively for 3 consecutive weeks, for a total of 18 sessions. Meanwhile, the rats in other groups were fixed at the same way but without moxibustion treatment, and during the dark phase all rats were blinded eyes by an opaque glove. Handing of mice and experimental procedures were in accordance with requirements of the Institutional Animal Care and Use Committee and this study was granted permission by the Ethics Committee of Chengdu University of Traditional Chinese Medicine (CUCM-2016-07).

Sample collection

The day following the end of moxibustion treatment, samples were collected at the corresponding time of ZT groups, such as the rats of 0 am ZT groups were administrated at 11 pm-1 am, 6 am ZT groups at 5 am-7 am, 12 N ZT groups at 11 am-1 pm, and 6 pm ZT groups at 5 pm-7 pm.

RA model: FCA-induced arthritis

Rats of RA model group, 5-7 am moxibustion and 5-7 pm moxibustion were administrated by subcutaneous injection of 0.15 ml FCA at right foot pad to establish RA animal model, meanwhile the rats of normal control group were injected 0.15 ml physiological saline (PhyS) at the same location of right foot.

Foot volume measurement

The body weight and right foot volume of each group rats were measured before the modeling (the first day of the experiment), after the modeling (the seventh day of the experiment), and after the weekly treatment (the 14th, 21st, and 28th days of the experiment) by using the self-made foot volume measuring device. First, fix the 20 ml syringe on the gantry and inject water (18 ml before modeling, 16-17 ml after modeling). Marked a line by the oily pen at 0.2 mm under the right knee, then placed the right foot into the water of a 20 ml syringe until the liquid level is parallel to the marked line, then the water is pumped with a 5 ml syringe connected to the rubber tube until 20 ml. The liquid level recess in the syringe is restored to the starting scale and the volume of water in the 5 ml syringe is read as the measured foot volume. In order to reduce the bias caused by subjective visual readings, each measurement person is fixed and the division of labor is clear.

Foot swelling index

The formula of [(Molding the volume of the foot after the mold-the volume of the forefoot)/ Modeling the volume of the forefoot = the swelling degree of the foot after modeling; (the volume of the foot after treatment-the volume of the forefoot before modeling)/the volume of the forefoot before the formation = the swelling degree of the foot after treatment] was adopted to calculates the degree of swelling of the foot at different time points.

RT-PCR

Total RNA were isolated from hypothalamus, hippocampus, and adrenal gland by Trizol (In-

Table 1. Primer list of real-time quantitative PCR

gene	Bidirectional primer sequence	Annealing temperature (°C)	Product length (bp)
β-actin (R)	F: 5'CGAGTACAACCTTCTTGCAGC3'	60	202
	R: 5'ACCCATACCCACCATCACAC3'		
REV-ERBα	F: 5'TGTCCGTATCAATCGCAAC3'	60	119
	R: 5'GCATTCGTTGCTTCTCTCTCT3'		
BMAL1	F: 5'CAGAAGCAAACTACAAGCCAA3'	60	99
	R: 5'GGTCACATCCTACGACAAACA3'		
CLOCK	F: 5'AAGATGACAAGGACAAAGCAA3'	60	165
	R: 5'TGCGTAAAAAATCAATGCTCT3'		
PER2	F: 5'TTTTTCTGCCGTGTCAGTGTT3'	60	213
	R: 5'GTTTGGTGTGTGGGTTGTTGT3'		

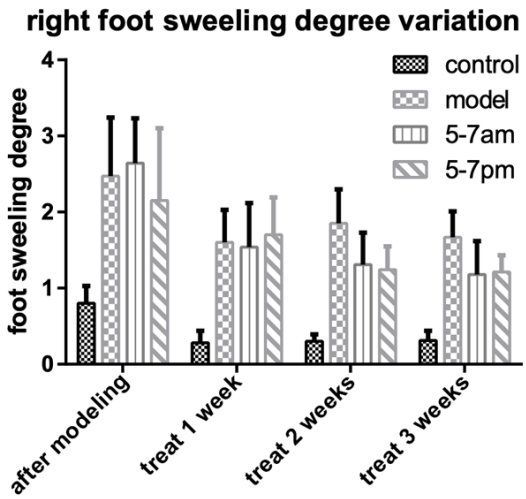

Figure 1. Right foot swelling in each group.

vitrogen) extraction and 1.5 μg of total RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription kit (Applied biosystems). Relative mRNA levels were determined using quantitative PCR and normalization to housekeeping gene. Primer sequences are available upon request. For analysis of gene expression, SYBR green gene expression assays were used for quantitative RT-PCR of REV-ERBα, CLOCK, BMAL1, and PER2. The primer sequences are shown in **Table 1**.

The gene-specific primer design was screened according to the mRNA sequences of rat β-actin and REV-ERBα in the NCBI database by Premier5 primer design software.

Statistics

All the data were calculated by SPSS 21.0 statistical software and presented as means ± SD. Data are presented as means + S.D. The differences were analyzed using Student's t-test or one-way ANOVA with multiple comparisons for assessment of more than two groups on GraphPad Prism software. Halberg cosiner software was used to analysis the circadian rhythm. $P<0.05$ and $P<0.01$ were considered as statistical significance. The symbol * denotes $P<0.05$ with ** for $P<0.001$.

Results

Moxibustion alleviates the swelling of RA-affected joint significantly

Shown as **Figure 1**, after modeling and 1 week of moxibustion treatment, compared with control group, the right foot of other three groups were significantly swollen, $P<0.01$, indicating that the adjuvant arthritis model was success. However, the swelling degree decreased 1 week later, even the $P>0.05$.

After 2 and 3 weeks of moxibustion treatment, compared with normal control group, the right foot of the model group was still swollen, $P<0.01$. Compared with the model group, the right foot swelling degree of the rats in both treatment groups was significantly decreased, $P<0.01$. However, there was no statistical difference between 5-7 am group and 5-7 pm group, $P>0.05$.

The degree of swelling of the RA joint is positively correlated with its inflammatory response, that is, the more severe the inflammation, the higher the degree of joint swelling. The results of this experiment show that the accumulation of moxibustion effect can significantly alleviate the swelling of RA-affected joints, and the joint morphology continues to improve with the

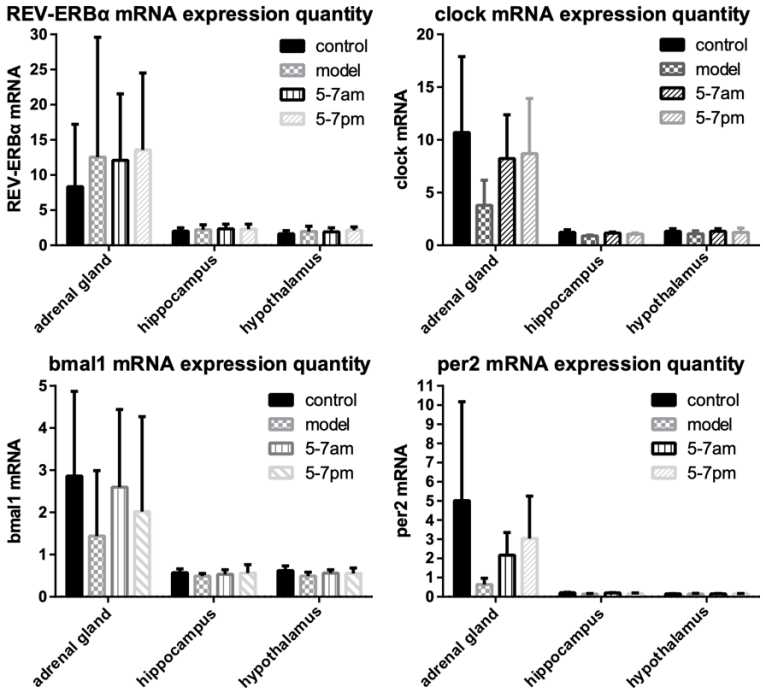

Figure 2. The relative expression quantity of core circadian genes mRNA in different tissues.

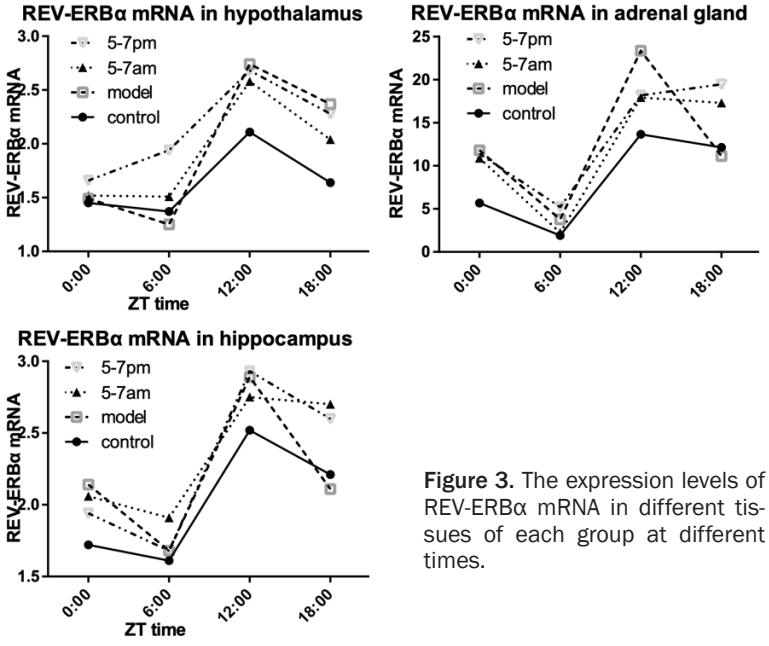

Figure 3. The expression levels of REV-ERBα mRNA in different tissues of each group at different times.

There were no significant differences between the moxibustion groups at different times. There may be tiny differences in the effects of moxibustion treatment on the macroscopic state of RA at different time, while there may be obvious differences in microscopic levels such as cytokines, hormone levels and genes. Therefore, further experiments will observe the effects of moxibustion on core clock genes in RA at different times.

*Core circadian genes highly expresses in adrenal gland, as the **Figure 2** show*

The relative expression quantity of core circadian genes mRNA were higher in the adrenal gland, while it was lower in the hippocampus and hypothalamus.

Compared with other groups, the relative expression of core circadian genes mRNA of model group were increased in different degree, especially CLOCK, BMAL1, and PER2 in adrenal gland.

The abundance of core circadian genes, REV-ERBα, CLOCK, BMAL1 and PER2 mRNA in different tissue samples were measured by the RT-PCR, illustrating the relative expressing quantity in adrenal gland, hippocampus and hypothalamus. The results of this experiment showed that the relative expression quantity of CLOCK, BMAL1 and PER2 in the hypothalamus of RA rats were not significantly different compared to those other groups rats. It was confirmed again that the expression level and rhythms of circadian clock genes in the central circadian clock were more stable than these in peripheral circadian clock.

extension of moxibustion session. Moxibustion may improve the morphological abnormalities of RA joints by inhibiting the secretion synthesis of inflammatory cytokines and alleviating the accumulation of pro-inflammatory factors in the synovial membrane of joints.

The expression levels of CLOCK and PER2 mRNA in hippocampus and adrenal gland of RA rats were significantly lower than those in normal rats, and moxibustion significantly up-regulated the expression of CLOCK and PER2 mRNA in hippocampus of RA rats. These results indicated that the expression of CLOCK and PER2 mRNA in peripheral tissues of RA rats is down-regulated, and the effect of RA on peripheral tissue biorhythm is more significant. That indicated moxibustion could notably increase the expression quantity of CLOCK and PER2 mRNA in hippocampus.

The REV-ERBα mRNA expressed differently at different times and usually had Diurnal oscillation, shown as the ***Figure 3***

The expression level of REV-ERBα mRNA in the adrenal gland, hippocampus, and hypothalamus of each group changed with time, and there were significant differences among each time points, $P<0.05$. In addition, the change trend of REV-ERBα mRNA of each group were almost consistent, and the peak time of expression was around 12:00, which appeared in the light phase, while the expression level in the dark phase was relatively low.

Circadian rhythm of REV-ERBα mRNA of model group in adrenal gland and hippocampus disappeared, but moxibustion recovered their circadian rhythm

Curves and polar coordinates of circadian variations in REV-ERB, 360° = 24 hours, 00:00 = 0°. (a) Curve of circadian REV-ERB changes for control, model, 5-7 am, and 5-7 pm; (b) Polar coordinate of circadian REV-ERB changes for control, model, 5-7 am, and 5-7 pm, respectively.

The results of this experiment showed that there was no significant difference in the expression of REV-ERBα mRNA in the hypothalamus, hippocampus and adrenal gland of each group, but the expression level of experimental RA rats was higher than that of the normal control group. Previous studies have confirmed that REV-ERBα is able to inhibit the secretion of inflammatory factors then relieve inflammation. Therefore, we speculated that the expression of REV-ERBα in different tissues of RA model rats may increase to varying degrees, which may be the results of body's stress response to inflammation. However, the magnitude of its REV-ERBα up-regulation is not sufficient to counter the severe inflammatory response of RA. Moxibustion has a tendency to up-regulate the expression of REV-ERBα in different tissues of RA rats, suggesting that moxibustion may inhibit the circulating levels of inflammatory factors by up-regulating the expression of REV-ERBα to play an anti-inflammatory effect role.

By analyzing the circadian rhythm of REV-ERBα mRNA in different tissues of each group, the expression of circadian clock receptor ---REV-ERBα mRNA in the hypothalamus of each group rats showed circadian rhythm ($P<0.05$). However, there were no diurnal oscillation in the adrenal gland and hippocampus of model group ($P>0.05$), while the moxibustion treatment recovered the circadian rhythm ($P<0.05$). As the **Figures 4**-**6** show, the peak phases of REV-ERBα mRNA expression notably shifted back to some extent, but they were approaching to the normal control group in the moxibustion groups. We found that the circadian rhythm in the hypothalamus of the model group was disordered, while the circadian rhythm in the hippocampus and adrenal gland disappeared. It can be seen that the loss or disorder of the circadian rhythm of REV-ERBα mRNA in different tissues of RA rats is an important basis of RA pathology rhythm, and its oscillation at the biological clock level changes significantly, which has a direct impact on the rhythm stability of various levels in the body environment. Therefore, we believe that the disappearance of REV-ERBα circadian rhythm may be an important cause of rhythm disorder of RA cytokines and hormone levels, which together form the pathological rhythm of RA. As for the reason of the circadian rhythm of REV-ERBα in the hypothalamus still exists, we speculated that its circadian rhythm as the central circadian clock SCN may be more stable than the peripheral circadian clock. The moxibustion has a significant effect on the regulation of REV-ERBα in RA rats, which not only restores the circadian rhythm of REV-ERBα mRNA in peripheral circadian clock, but also regulates the rhythm parameters of REV-ERBα in the central circadian clock in the normal direction. Consequently, Moxibustion can adjust the circadian rhythm of its biological clock gene to maintain the stability of the environment rhythm for central clock and peripheral clock.

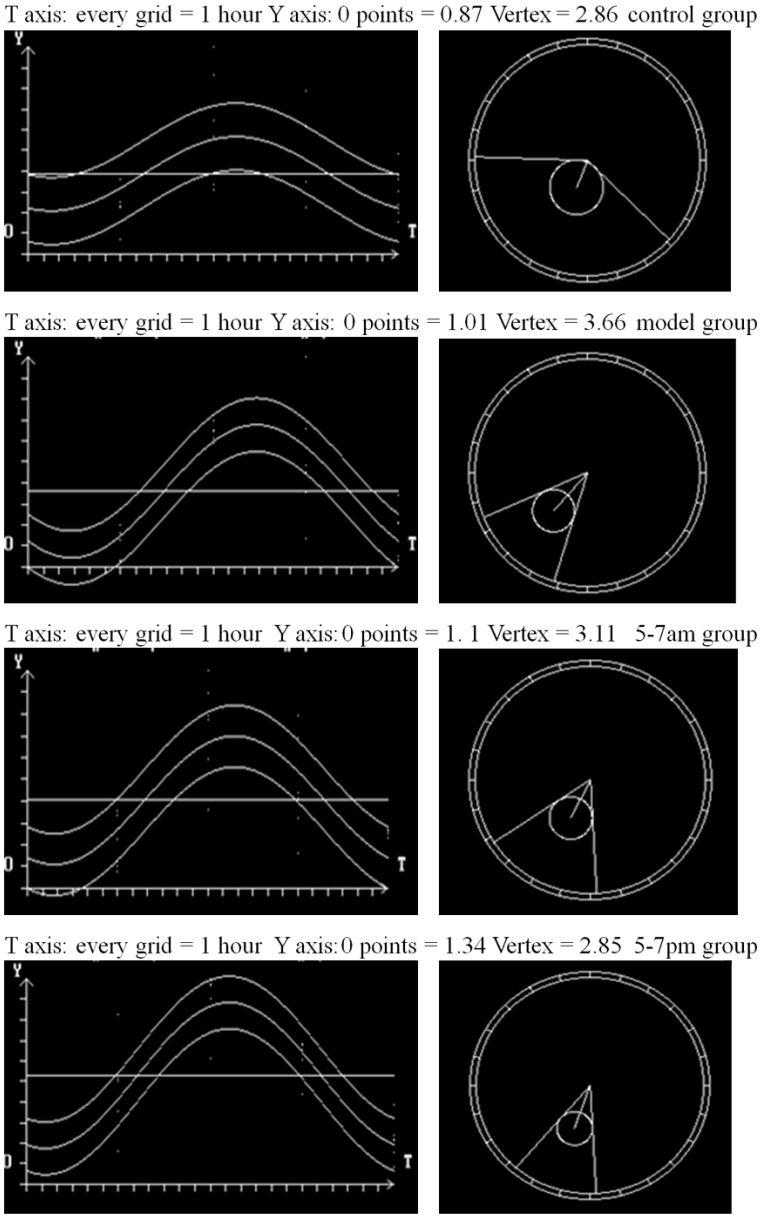

Figure 4. Cosine fitting curve and polar coordinates of REV-ERBα mRNA in hypothalamus of each group rats.

Discussion

In RA, key clinical manifestations show a circadian rhythm, with more prominent joint swelling, stiffness, and pain occurring in the early morning and relative relieved in the afternoon. Clinical studies have confirmed that the joint stiffness, swelling, and pain of RA patients is most serious during 4-8 am, while most relieved at about 4 pm [2]. Previous research found that the circadian rhythm of RA symptoms is contributed to the diurnal variation of pro-inflammatory cytokines and hormones closely related to RA, such as GCs, IL-1, IL-6, and TNF-α et al [3, 13]. For example, IL-1β, IL-6, and TNF-α are all elevated in sera of rheumatoid patients reaching the peak levels in early morning [9, 10]. Specifically the serum TNF-α exhibit a delayed secretion rhythm in the patients with RA as compared with healthy controls, being highest at 6 am and remained upregulated until 10 am, which coincides with the signs of morning stiffness in RA [14].

Circadian clock regulates numerous physiological processes that vary across the day-night cycle, and plays a vital role in RA. The central clock lies in the SCN of the brain and maintains essential synchrony of peripheral tissue clocks via neural and humoral mediators. Virtually all cells in the body express components of the cellular circadian clock and are capable to sustain circadian oscillations. Previous studies considered that there is also a bidirectional communication between the circadian clock system and the immune system involved in RA pathogenesis. Circadian disruption alters disease rhythmicity, for instance the signs and symptoms of RA can be modulated by the circadian clock, while RA also significantly disturbs the biological clock of host [15, 16]. Mounting evidence indicates that shift work is associated with a wide variety of adverse health consequences [17], a study in 2010 confirmed that shift work has previously been associated with increased RA risk in females [18]. In conclusion, in addition to the influence of pro-inflammatory cytokines and hormone levels on the pathophysiology of RA, the disorder circadian clock system is also deeply involved in the pathogenesis of RA. Recent

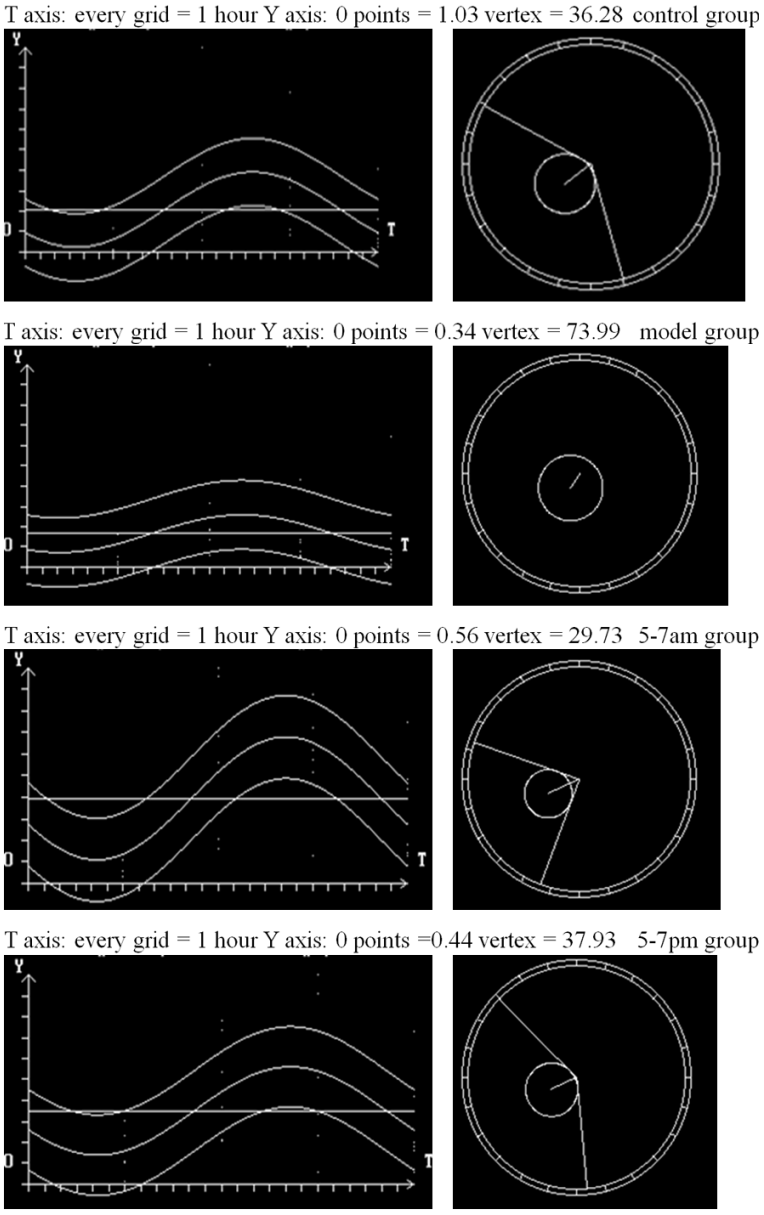

Figure 5. Cosine fitting curve and polar coordinates of REV-ERBα mRNA in adrenal gland of each group.

studies have confirmed that RA inflammation is interacted with the circadian clock, and the disturbed biological clock affects the immune system and thus negatively participates in the pathological mechanism of RA [7, 15, 16].

As the key components of circadian clock system, core clock genes play a key role in maintaining circadian rhythm. Lots of clock genes widely exist in the inflammatory tissues and cells of RA organism, they are transmitted to the central clock through signal transduction pathways, which in turn regulate the expression and activation of genes and proteins which exact an important role in RA inflammation. That means core clock genes adjust the circadian clock system, while inflammation directly changes the cell expression of core clock genes [3, 6, 7, 19-27]. Therefore, scholars believe that circadian rhythm of cytokine and hormone levels in RA patients may be closely related to the changes of clock gene expression. Clock genes regulate the circadian clock system. For example, wild mice maintain regular circadian rhythm in the absence of light, while mice which is knocked out cry1 and cry2 is unable to maintain behavioral rhythm in the dark [28]. To date, it has been confirmed that CLOCK, BMAL1, CRY and PER are mammalian core clock genes, which also deeply involved in the pathogenesis and pathological process of RA [15]. RA inflammatory factors interact with clock genes: clock genes affect the circulating concentration and rhythm of RA inflammatory factors, while RA-related inflammatory factors abnormalities can disrupt the expression of clock genes in turn.

The nuclear receptor REV-ERBα, a powerful transcriptional repressor, links circadian and inflammations through the regulation of immune system function, of which REV-ERBα is more highly functional [29]. In 1998, REV-ERBα was noted to one of the genes that oscillates within the circadian transcriptome of mammalian cells cycling in tissue culture. The nuclear receptor REV-ERBα acts in a tissue-specific manner to regulate circadian rhythms as well as metabolism. In addition, Bio-clock system and RA inflammatory pathologies intertwine, and indeed REV-ERB regulate metabolic function in many tissues, thus the nuclear REV-ERBα is a key transcriptional link between them

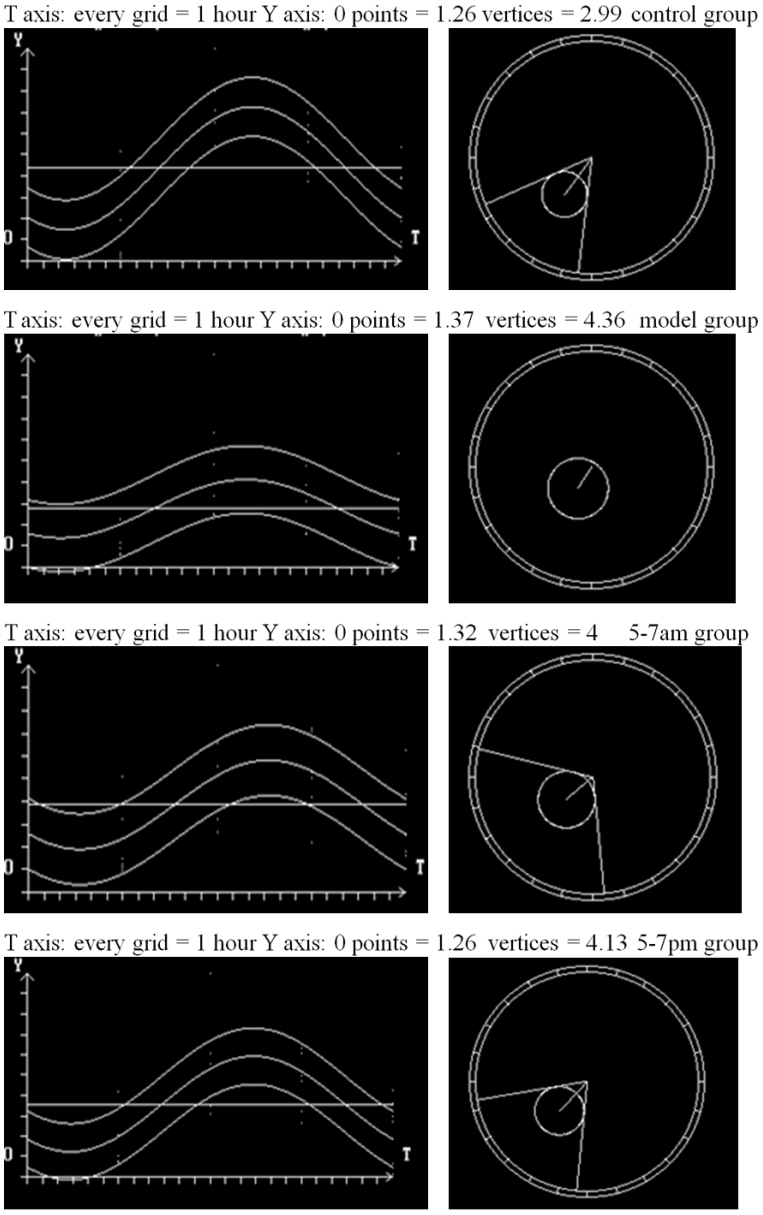

Figure 6. Cosine fitting curve and polar coordinates of REV-ERBα mRNA in hippocampus of each group rats.

[30-33]. As known, it is able to modulate the rhythmicity of additional circadian regulators, including CLOCK, Cry1, and thus has a major influence on the cell-autonomous molecular timing system [33]. Previous study showed the circadian expression of REV-ERBα in RA rats peaks between ZT12-ZT16, which is anti-phase to the circadian rhythm of key pro-inflammatory cytokines related to RA, such as IL-6 and TNF-α [34].

Consequently, we proposed that moxibustion may regulate the circadian rhythm of RA via benign adjust the expression and diurnal variation of core clock genes. The results of this study show that moxibustion could benign the circadian rhythm of REV-ERBα mRNA in the central clock SCN and peripheral circadian clock, and to regulate the circadian rhythm at the molecular clock level. The biological clock gene regulates the circadian clock system at the molecular level, and it dominates the time rhythm of various levels of the body's nerves and endocrine. Therefore, moxibustion is likely to achieve a benign regulation of RA by regulating the circadian rhythm of the circadian clock nuclear receptor in different tissues of RA rats. The purpose of circadian rhythms of cytokines and hormone levels is to adjust the pathological rhythm of RA to exert its anti-inflammatory rhythmic effect.

However, there are some limitations of this work. First of all, the circadian rhythm REV-ERBα mRNA measured in this study only, maybe we need to analysis more core clock genes such as CLOCK, BMAL1, PER and CRY which related RA closely in order to discover more evidence to support the conclusion. And we will do this job in the further study more carefully. For another limitation, the definite pass-way of how clock genes influence the pro-inflammatory cytokines of RA is still unclear, so there is not enough evidence for moxibustion benign regulate circadian rhythm of RA.

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Disclosure of conflict of interest

None.

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